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**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant	: Usdin et al.
Appl. No.	: 10/014,162
Filed	: December 11, 2001
For	: PARATHYROID HORMONE RECEPTOR LIGANDS
Examiner	: Romeo, David S.
Group Art Unit	: 1647

**DECLARATION UNDER 37 CFR 1.132 OF TED B. USDIN, M.D., Ph.D.**

I, Ted. B. Usdin, M.D., Ph.D., do hereby declare:

1. I am a named inventor of the above-identified application. A true and correct copy of my Curriculum Vitae is attached as Exhibit 1.

2. I understand that the Patent Office takes the position that it would have been obvious to one of ordinary skill in the art at the time of the invention to make an isolated or purified peptide that is a PTH2 receptor ligand, as taught by Usdin et al., 1997, Endocrinology 138: 831, and to modify that teaching by using the PTH2 receptor, as taught by Usdin et al., 1995, J. Biol. Chem. 270: 15455, for the affinity purification of its ligand, as taught by Park in USP 5,194,375 and Angal S & Dean PDG, "Purification by exploitation of activity" Chapter 5, In, Protein Purification Methods: A Practical Approach, Harris ELV & Angal S (Eds.), September 1989, IRL Press, Oxford, UK, page(s) 245. This is incorrect. It would have been impractical, if not impossible, to use affinity purification because of the chemical nature of the PTH2 receptor. The PTH2 receptor is a glycosylated membrane embedded protein. It is a member of a large class of proteins referred to in the scientific literature as seven transmembrane domain receptors, because it contains seven distinct hydrophobic domains that span the plasma membrane of cells in which it is expressed, the transmembrane domains are separated from each other by hydrophilic domains that extend alternately into either the cytoplasm or extracellular space. The protein must be embedded in an appropriate lipid membrane in the correct

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conformation to bind its ligand. Members of this class of protein are also referred to as G-protein coupled receptors because they interact with members of a family of heterotrimeric GTP-binding proteins. In order to have high affinity for an agonist ligand, coupling to an appropriate combination of members of the heterotrimeric GTP-binding protein family is required. To the best of my knowledge no one has successfully developed an affinity matrix from a seven transmembrane domain receptor. This is an approach that I considered during work on the purification of TIP39 and rejected for several reasons. Methods for extracting this type of receptor from membranes in which they are normally expressed, and binding them to a matrix that could be used in affinity purification, in a manner that retains sufficient affinity for an agonist ligand, have not been established as a general practical approach, or in fact demonstrated for any member of this family. The receptor in the cited reference to Park, USP 5,194,375, is in an entirely different class and has a different membrane topology. A second approach to using the PTH2 receptor as an affinity support that could be considered would be to express it at high levels in a cell by means of transfection of the cells with a cDNA encoding the receptor, and then to use these cells, or membranes from these cells, as an affinity matrix without extracting the receptor. Again, this approach has not been established for this class of receptor. I considered attempting to develop the methodology during work on the ligands purification and rejected it as being impractical. A very high level of expression would be required to create a sufficient density of receptor. My prior experience with very high level receptor expression for other projects, and the published literature, show that once a very high expression density is achieved with this class of receptor, which itself can be a significant challenge, the receptor will be present in excess over the corresponding GTP-binding proteins, and thus the majority of the receptor molecules will have low affinity for the agonist. In addition, methods for using such cells, or plasma membranes prepared from such cells, as an affinity matrix have not been established. There are a number of handling procedures that would need to be developed, and which would likely make the process impractical.

3. Our process for purification was empirical and thus nonobvious because we knew very little about the composition of the ligand before we completed the purification. The essence of the chromatographic purification that we performed is to perform a series of chromatographic steps in which the activity of interest elutes in a different fraction from other components of the extract, and to combine a sufficient number of such steps that the material of interest elutes in a different fraction than essentially all other components of the extract, so that it becomes a sufficiently pure species that its sequence can be determined by chemical methods. Without knowing the composition of the material the design of chromatographic methods becomes empirical. It takes someone with expert skill to develop a sufficient number of chromatographic steps that distinguish between the activity of interest and all of the other components of the original extract, in a practical amount of time and with a finite amount of starting material. In other words, step number one may separate the activity from contaminants A through J, but leave it mixed with K through Z. A chromatographic step that uses a somewhat different principle must be then developed that separates the activity from K through P, and then another different procedure, etc. Each step incurs significant losses of material, and special handling procedures to minimize the loss and retain the activity needed to be developed. In addition each of the chromatographic procedures must yield material that can be evaluated by the bioassay being used. The bioassay used functional activation of the PTH2 receptor and measurement of a second messenger (cyclic adenosine monophosphate) that accumulated in the cells in response to activation of the PTH2 receptor in living cells. Material from many standard purification procedures either poisons cells or causes non-specific stimulation of cAMP accumulation. The selection of methods to avoid both of these, and to create a series of chromatographic steps with differing selectivity, are non-obvious.

4. While it turned out that we purified an activity from bovine hypothalamus that had the properties suggested by our original pre-patent application publication, Usdin et al., 1997, Endocrinology 138: 831, there are ways that the activity detected in the crude extract could have turned out not to be a unique ligand. For instance, there could have been an inhibitor in the hypothalamic extract that inhibited activation of the PTH1 receptor more than it inhibited the

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PTH2 receptor, which in combination with parathyroid hormone instead of a unique ligand created the published results. There were prior publications that claim the presence of parathyroid hormone in the hypothalamus, e.g., Nutley et al., 1995, Endocrinology 136: 5600 (attached as Exhibit 2), Fraser et al., 1990, Endocrinology 127: 2517 (attached as Exhibit 3); and Balabanova et al., 1986, Klin Wochenschr 64: 173 (attached as Exhibit 4).

5. An additional argument that one with "ordinary skill" could not have purified the activity simply based on the information in the original paper in Endocrinology is based on the failed attempt of others. Following that publication a group of investigators at Harvard University, in the Massachusetts General Hospital Endocrinology Unit, attempted to follow up and complete the purification (in competition with me) and failed to do so. This group has considerable expertise, and includes the world experts on parathyroid hormone and parathyroid hormone receptors. The documentation of this failed attempt is an abstract for a meeting that the investigators published in which they reproduced my published work, Nineteenth Annual Meeting of the American Society for Bone and Mineral Research, Cincinnati, Ohio, September 10-14, 1997, Abstract # S368 (attached as Exhibit 5). In combination with their lack of further publication on the topic until after my publication of the sequence this presents an argument to those with an understanding of the field that they failed in an attempt to purify the material.

6. Another argument based on professional recognition comes from a report that was written when I was evaluated for scientific tenure at NIH. The purification of the peptide was one of the major factors in the decision to grant tenure. Attached as Exhibit 6 is a document that is the first page (which includes the executive summary and parts of the comments of one of the reviewers) of the report of the Board of Scientific Councilors that evaluated my work and made the tenure recommendation. They point out the significance of the accomplishment, how it could easily have failed, and how I was scientifically creative in accomplishing it.

7. I understand that the Patent Office holds that there is no evidence of record that PTH2 receptor ligands are especially variant. This is incorrect. Usdin, T.B., 2000, TIPS 21: 128, which I understand is of record, and which I wrote around the 15 June 1999 filing date of the



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present application, aligns PTH, which is a PTH2 receptor ligand, from several species, with PTHrP, which is a PTH1 receptor ligand, from several species, and TIP39, which is a PTH2 receptor ligand, from bovine species. Figure 1 in Usdin, T.B., 2000, TIPS 21: 128 shows that, while nine of the 39 residues of TIP39 are identical to bovine PTH, turning to PTH itself, of the first 34 residues (which possess full activity), 20 have been demonstrated to be variant:

Bovine TIP39	SLALADDAAFREHARLLAAEERHNLNSYM---HKLLVLDAP
Bovine PTH	AVSEIQFMHNLCKHLSSMERVEWLRKKLQDVHNFVALGAS...
Pig PTH	SVSEIQLMHNLCKHLSSLERVEWLRKKLQDVHNFVALGAS...
Dog PTH	SVSEIQFMHNLCKHLSSMERVEWLRKKLQDVHNFVALGAP...
Human PTH	SVSEIQLMHNLCKHLNSMERVEWLRKKLQDVHNFVALGAP...
Rat PTH	AVSEIQLMHNLCKHLASVERMQWLRKKLQDVHNFVSLGVQ...
Chicken PTH	SVSEMQLMHNLCGEHRHTVERQDWLQMKLQDVHNSALEDART...
Fugu PTHrP	SVSHAQLMHDKGRSLQEFRRHMLH-LLAEVHT-ABY...
Chicken PTHrP	AVSEHQLLHDKCKSIQDERRRFFLHHLIAEIHT-ABIRAT...
Human PTHrP	AVSEHQLLHDKCKSIQDERRRFFLHHLIAEIHT-ABIRAT...
Dog PTHrP	AVSEHQLLHDKCKSIQDERRRFFLHHLIAEIHT-ABIRAT...
Mouse PTHrP	AVSEHQLLHDKCKSIQDERRRFFLHHLIAEIHT-ABIRAT...
Rat PTHrP	AVSEHQLLHDKCKSIQDERRRFFLHHLIAEIHT-ABIRAT...
	1 5 10 15 20 25 30 35 40

*trends in Pharmacological Sciences*

Thus there is considerable variation in one PTH2 receptor ligand. Based on the similarity between the PTH2 receptor and the PTH1 receptor (both activated by PTH, overall approximately 50% sequence similarity, regions of very high sequence similarity) and the similarity between TIP39 and PTH it can be considered that TIP39 is a member of a peptide family with PTH and PTHrP. PTH and PTHrP have indistinguishable binding to and activation of the PTH1 receptor. There is even further variation than that for PTH when all of the species of PTH and PTHrP that were known to activate that PTH1 receptor are considered. Thus because of the relationship of TIP39 to PTH and to PTHrP one could reasonably anticipate natural variation in TIP39 from different species, and further that artificially induced variation in many residues within the sequence would be tolerated with little effect on binding to the PTH2 receptor. Our discovery of TIP39 in combination with the comparison with the sequences of PTH and PTHrP demonstrates that considerable variation in the sequence of PTH2 receptor ligands, and, additionally, PTH1 receptor ligands, is tolerated, that certain residues are likely to be critical, and that the three dimensional conformation induced by others, but not their particular

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side chains are important. It should be possible to tease these possibilities apart by studying appropriate peptide modifications and their effect on PTH2 and PTH1 receptor binding activity.

8. Additionally, the post-filing date scientific literature of Hansen et al., 2002, J. Endocrinol. 174: 95 (attached as Exhibit 7) identified and characterized the human and mouse genes encoding TIP39 and Papasani et al., 2004, Endocrinology 145: 5294 (attached as Exhibit 8) identified and characterized the zebrafish and pufferfish genes encoding TIP39. Figure 4 of Papasani 2004 aligns TIP39 amino acid sequences from zebrafish, pufferfish, human, and mouse:

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                                ↓
f TIP39  Msfsksddata                                     aK
z TIP39  Ma  LSLPPRPALLFLVLMSVTLMasafpqpqlrplqsnLPAIGqedsK
h TIP39  METRQVSRSPRVRLLLLLLLLLLVVPWGVrtASGVA      LPPVGV
m TIP39  METCQMSRSPRERLLLLLLLLLVVPWGVtgpASGVA      LPLAGV
          *      *      ** *::: : :      ** *

f TIP39  qdnWDVFFPSLFLHNWKIQTMSAPTLEAAAASNKRLVQQGWL FGPQRME
z TIP39  gegWEVVYPSISLRDWSIQMLTAPDFGAAKTGREQLVADDWLpLSQSOME
h TIP39          LSLR          PPG
m TIP39          FSLR          APG
          *              *

                                U+1
f TIP39  TSLDGVLPOEWASQSGgmVKRNMVADDAAFREKSKMLTSMERQKWLNSY
z TIP39  EELVKGWTGDWPSRVGhgqKRNIIVADDAAFREKSKLLTAMERQKWLNSY
h TIP39  RAWADPATPrpRRSLALADDAAFRERARLLAALERRHWLNSY
m TIP39  RAWAGLGSPlsRRSLALADDAAFRERARLLAALERRRWLDSY
          *      :*:::*****:::***:***

f TIP39  MQKLLVVNSa 123
z TIP39  MQKLLVVNSk 157
h TIP39  MHKLLVLDAP 100
m TIP39  MQKLLLDAP 100
          *:***:::

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When comparing man and mouse, the first 23 aa of the TIP39 sequence, thought to contain the PTH2 receptor activation site, are identical, while the 16 aa C-terminal portion showed a higher degree of diversity (75% aa identity). By comparison, alignment of TIP39 aa sequences from zebrafish, pufferfish, human, and mouse revealed that, of the first 23 amino acids, 11 amino acids were identical, while of the last 16 amino acids, 8 were identical, resulting overall in about 50% aa identity. Thus, the post-filing date art confirms that TIP39 tolerates considerable amino acid sequence variation.

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I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Respectfully submitted,

Dated: 8/4/2005

By:   
Ted. B. Usdin, M.D., Ph.D.

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# **EXHIBIT 1**



Fred B. Usdin, M.D., Ph.D.

## Curriculum Vitae

### Address

Laboratory of Genetics, National Institute of Mental Health  
35 Convent Dr., Bethesda, Maryland 20892-4094  
telephone: 301-402-6976, fax: 301-402-0245  
e-mail: [usdint@mail.nih.gov](mailto:usdint@mail.nih.gov)

### Current Position

Senior Investigator, Laboratory of Genetics  
National Institute of Mental Health, National Institutes of Health

### Education and Professional Experience

- |                 |  |
|-----------------|--|
| 1974-1978       | B.A., Biophysics<br>Johns Hopkins University Baltimore, MD.  |
| 1978-1979       | Research Assistant<br>Johns Hopkins Univ., Baltimore, MD<br>Supervisor: Dr. Solomon Snyder   |
| 1979- 1986      | Medical Scientist Training Program<br>Washington University , St. Louis, Mo.<br>M.D., Ph.D<br>Doctoral Thesis: Identification of a protein which stimulates acetylcholine receptor incorporation in chick myotubes grown in culture: a possible synaptogenic factor<br>Advisor: Dr. Gerald Fischbach |
| 1986-1990       | Residency in Psychiatry, Stanford University, Stanford, CA.<br>Residency Director: Dr. Roy King  |
| 1990-1993       | National Research Council Fellow, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD<br>Supervisor: Dr. Michael Brownstein  |
| 1993-1997       | Senior Staff Fellow, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda MD<br>Supervisor: Dr. Michael Brownstein  |
| 1997 to 2002    | Investigator, Laboratory of Genetics, National Institute of Mental Health Bethesda MD  |
| 2002 to present | Senior Investigator, Laboratory of Genetics, National Institute of Mental Health, Bethesda MD  |

**Awards**

O'Leary Prize in Neuroscience Research, Washington University, 1985.

Dana Fellowship, Stanford University, 1988-1990.

NARSAD Young Investigator Award, 1992-1994.

**External Invited Presentations**

- 1992 NARSAD Annual Meeting
- 1993 Georgetown Univ. Dept. Pharmacology  
Oxygen Club of Greater Washington D.C.
- 1994 Stanford Univ. Dept. Psychiatry  
Muscular Dystrophy Assoc., Phoenix AZ
- 1995 Medical College of Virginia, Dept. Pharmacology
- 1996 Div. Bone and Mineral Metabolism Beth Israel Hospital, Boston MA  
Korean Society of Medical Biochemistry and Molecular Biology, Seoul Korea  
R.W. Johnson Pharmaceutical Research Institute, NJ
- 1998 American Society of Nephrology, Philadelphia PA
- 1999 Lilly Pharmaceutical Co, Indianapolis IN  
Fourth International Conference on New Actions Of Parathyroid Hormone, Malta  
Merck Research Laboratories, NJ  
Endocrine Unit, Massachusetts General Hospital  
Dept. Psychiatry, Univ of Calif. San Francisco  
Amgen, Inc.
- 2000 American Society for Bone and Mineral Research, Toronto Canada  
Biomeasure, Inc. MA
- 2001 Dept. Psychiatry, Univ of Calif. San Francisco  
Adolor, Inc., Malvern, PA.
- 2003 Astrazeneca, Inc., Montreal, Canada  
Dept. Anatomy, Semmelweis University, Budapest Hungary  
Dept. Psychiatry, Univ. Bonn, Bonn Germany
- 2005 Center for Neuroscience, Louisiana State University, New Orleans, LA

**Current Committee Service**

NIMH Animal Care and Use Committee

NIMH Transgenic Mouse Core Facility Users Committee

Trans-Institute Neuroscience Lecture Series Speaker Selection Committee

NINDS Light Imaging Facility Oversight Committee

NMH Technology Transfer Advisory Committee

Porter Neuroscience Research Center Animal Facility Users Committee

**Publications:**

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# **EXHIBIT 2**

# Sequence Analysis of Hypothalamic Parathyroid Hormone Messenger Ribonucleic Acid\*

M. T. NUTLEY†, S. A. PARIMI, AND S. HARVEY

Department of Physiology, University of Alberta, Edmonton, Canada T6G 2H7

## ABSTRACT

PTH-like peptides and messenger RNA (mRNA) have recently been detected in neural tissues, but it is uncertain whether this reflects the transcription of the PTH gene or that of a closely related gene. This possibility has, therefore, been investigated.

PTH-like complementary DNA (cDNA) moieties of predicted size were readily generated from reverse transcribed brain (hypothalamic and extrahypothalamic tissue) and pituitary RNA, using polymerase chain reaction (PCR) with three sets of overlapping oligonucleotide primers designed to amplify PTH cDNA fragments of 285, 372, and 459 base pairs (bp). PCR reamplification of the largest hypothalamic moiety with an internal set of primers also generated a cDNA fragment of the predicted size (372 bp). Restriction endonuclease digestion with *Bst*NI cleaved the largest hypothalamic cDNA moieties into smaller fragments of 217 and 242 bp, identical to the cleavage of parathyroidal PTH cDNA. Rapid amplification of cDNA ends of the 3'-flanking cDNA sequences also produced hypothalamic and extrahypothalamic cDNA moieties identical in size (499 bp) to parathyroidal PTH cDNA. Southern analysis of these PCR and rapid amplification of cDNA end cDNA fragments further indicated homology

with PTH cDNA. This homology was subsequently confirmed by nucleotide sequencing, which demonstrated complete homology between the neural and parathyroidal cDNA fragments. This homology extended over 673 bp (spanning nucleotides 31–709 of PTH cDNA), encompassing 95% of the entire parathyroidal PTH cDNA. The mRNA for this gene, determined by Northern blotting with a riboprobe for PTH mRNA, was of identical size to the parathyroidal PTH, but its abundance in brain was less than 0.01% of that expressed in the parathyroid glands. This transcript was not, however, detected in liver. The translation of this moiety in hypothalamic tissues was indicated by the presence of a protein in the rat hypothalamus that was immunoreactive with PTH-(1–84) antiserum and of comparable size to that in parathyroidal tissue. The abundance of this protein in hypothalamic tissue was approximately 0.25% of that in the parathyroid glands, suggesting tissue-specific differences in its rate of synthesis, processing, or degradation.

These results, therefore, demonstrate that the brain is an extrapathyroidal site of PTH gene expression and suggest autocrine or paracrine roles for PTH in neural function. (*Endocrinology* 136: 5600–5607, 1995)

PTH-LIKE immunoreactivity is present in extracts of the rat hypothalamus (1) and sheep brain (2, 3). The immunoreactivity in these tissues is associated with a heat-stable nondialyzable peptide that coelutes with authentic PTH-(1–84) after HPLC fractionation (1). The blood-brain barrier has been considered to be impermeable to PTH in systemic circulation (4, 5), and the brain may, therefore, be an extrapathyroidal site of PTH production. This possibility is supported by the localization of PTH immunoreactivity within perikarya in discrete rat hypothalamic nuclei (6), in which PTH-like messenger RNA (mRNA) was detected by *in situ* hybridization (7). It is, however, still uncertain whether the PTH-like immunoreactivity and PTH-like mRNA in the brain reflect the extrapathyroidal transcription of the PTH gene or the expression of a closely related gene.

The PTH-like mRNA in the rat hypothalamus was found, by Northern blotting with a PTH riboprobe, to be of comparable size to the RNA moiety in parathyroidal tissue (7). Some sequence homology with the parathyroidal transcript was also indicated by the generation of a 384-base pair (bp)

reverse transcriptase-polymerase chain reaction (RT-PCR) complementary DNA (cDNA) moiety of predicted size, identical to parathyroidal PTH cDNA (7). This PTH-like transcript was, however, of low abundance, and the cDNA fragment generated by RT-PCR was only distinguishable after a booster PCR reamplification. It is, therefore, possible that this cDNA moiety was a PCR artifact, reflecting the illegitimate transcription of a constitutively expressed gene (8). It may also be possible that the "sticky end" PCR primers chosen for this study (7) simply lacked sufficient homology to the PTH gene, thus reducing the efficiency of PCR amplification. Therefore, the abundance of PTH-like mRNA in the rat brain and its sequence homology with parathyroidal PTH mRNA were determined in the present study.

## Materials and Methods

### Tissue preparation

Parathyroid glands, hypothalami, extrahypothalamic brain, pituitary glands, and liver tissues were rapidly dissected from 3-week-old (200–250 g) male Sprague-Dawley rats, immediately frozen in liquid nitrogen, and stored at –80°C before analysis. The hypothalami were excised from the rest of the brain through four perpendicular cuts in the shape of a diamond, each approximately 2.4 mm lateral and 3.5 mm dorsal of the midline (9).

### RNA preparation

Total cellular RNA was isolated from tissue extracts using RNA NOW (Bio/Can Scientific, Mississauga, Ontario, Canada). Briefly, after ho-

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Address all correspondence and requests for reprints to: Dr. Steve Harvey, Department of Physiology, Faculty of Medicine, University of Alberta, 7–55 Medical Science Building, Edmonton, Alberta, Canada T6G 2H7.

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† Recipient of a Natural Sciences and Engineering Research Council Studentship.

mogenization in RNA NOW, 0.2 ml chloroform was added to each sample and then shaken for 20 sec. After resting on ice for 5 min, the samples were centrifuged ( $10,000 \times g$  for 10 min at 4°C), and the RNA precipitates were washed twice with (1 ml) 75% (vol/vol) ethanol and centrifuged ( $5000 \times g$  for 5 min at 4°C) before resuspension in diethylpyrocarbonate-treated water. The purity and amount of RNA extracted were assessed spectrophotometrically at 260 nm after electrophoresis in ethidium bromide-stained 1% (wt/vol) agarose minigels (10). Total RNA was used to prepare polyadenylated [poly(A)<sup>+</sup>] mRNA using the polyATtract mRNA isolation system (Promega, Madison, WI).

### RT-PCR

The cDNA sequence of the neural PTH transcripts was determined by RT-PCR (11). Total (0.2–1.0 µg) or poly(A)<sup>+</sup> RNA (1 µg) from extracted tissue was reverse transcribed with Superscript (100 U; BRL, Burlington, Canada) in the presence of a 50-pmol 3'-oligomer rat (r) PTH antisense primer (MTN; 5'-GCACGGTCTAGAATACGTCAGCATTTA-3'), based on the known sequence of rPTH cDNA (12, 13), or a 100-pmol oligodeoxythymine primer (Boehringer Mannheim, Montreal, Canada), excess deoxynucleotides [10 mM each of deoxy (d)-ATP, dCTP, dGTP, and dTTP; Boehringer Mannheim], and 5 × RT buffer (BRL, Gaithersburg, MD). The reactions were diluted with double distilled water (50:1, vol/vol), and an aliquot of each (0.5% of total volume) was added to a PCR mixture containing one of three overlapping oligonucleotide primers (MTN1, 5'-AAGAGAGCTGTCAGTGAATACAGCTT-3'; MTN3, 5'-AGTCCAGTTCATCAGCTGCTGGCTTA-3'; MTN2, 5'-ATGATGCTGCAAGCACCATTGGCTAAG-3; Nucleotide Synthesis Laboratory, University of Alberta, Edmonton, Canada). These primer sets were based on the nucleotide sequence of the rPTH gene and were designed to generate fragments of 459 bp (MTN3; spanning nucleotides 36–2082 and cDNA residues 36–494, from the first, second, and third exons), 372 bp (MTN2; spanning nucleotides 1600–2082 of the PTH gene and cDNA residues 123–494, from the second and third exons), and 285 bp (MTN1; spanning nucleotides 1797–2082 of the gene and cDNA residues 210–494, from the third exon; Fig. 1a). A common 3'-oligomer rPTH antisense primer (MTN) was used in each primer set. The PCR mixtures contained 15 pmol of each oligonucleotide primer set, excess deoxynucleotides (200 µmol of each), 1 × PCR buffer [80 mM KCl, 16 mM Tris-Cl (pH 8.4), 1.5 mM MgCl<sub>2</sub>, and 0.1% Triton X-100], and *Thermus aquaticus* (Taq) DNA polymerase (5 U; Promega). The mixtures were overlaid with mineral oil (2 drops) and denatured at 94°C for 2 min before 35 cycles of denaturing (92°C for 1 min), annealing (50°C for 1.5 min), and extension (72°C for 2 min), ending with a final extension (72°C for 10 min) in a genetic thermal cycler (MJ Research, Watertown, MA). Reaction products were analyzed by electrophoresis in ethidium bromide-stained 1.5% (wt/vol) agarose gels and compared with DNA mol wt markers  $\phi$ X174 RF DNA/*Hinf*I (Promega).

### Nested PCR

The large PCR fragment, generated by the MTN3 primer set, was excised from the agarose gel and purified using a cDNA gel purification kit (Gene Clean II, Bio/Can Scientific, Mississauga, Ontario, Canada), according to the manufacturer's instructions. cDNA aliquots were then overlaid with equal volumes of mineral oil and reamplified by heating to 94°C for 2 min before 30 cycles of annealing (50°C for 1 min), extension (72°C for 30 sec), and denaturation (94°C for 30 sec), followed by a final extension (72°C for 10 min) with the internal (MTN2) oligonucleotide primer set.

### Restriction endonuclease digestion

Sequence homology with the PCR-generated cDNA moieties (MTN3/MTN) was determined by restriction endonuclease digestion (1 h at 37°C) with *Bst*NI (5 U/µg DNA; Boehringer Mannheim), which would result in PTH cDNA residues of 217 and 242 bp (13). Digestion products were identified after electrophoresis in ethidium bromide-stained 1.5% (wt/vol) agarose gels.

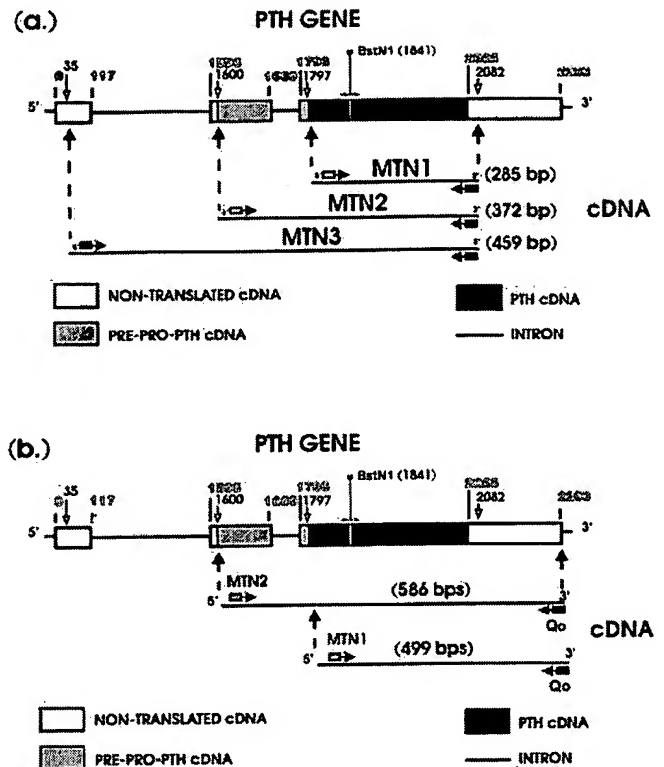


Fig. 1. PCR and 3'-RACE primer map demonstrating the sites of primer annealing and endonuclease digestion on the PTH gene. Primer sets were based on the nucleotide sequence of the rPTH gene (12). Three overlapping PCR primers (a) generate fragments of 459 bp (MTN3; spanning 36–2082 bp; first, second, and third exons), 372 bp (MTN2; spanning 1600–2082 bp; second and third exons), and 285 bp (MTN1; spanning 1797–2082 bp; third exon only). All primer sets use a common 3'-oligomer rPTH antisense primer (MTN). 3'-RACE primers (b) generate fragments of 586 bp (MTN2 and Q<sub>o</sub>; spanning 1600–2082 bp; second and third exons) and 499 bp (MTN1 and Q<sub>o</sub>; spanning 1803–2082 bp; third exon only).

### 3'-Rapid amplification of cDNA ends (3'-RACE)

3'-RACE (14) was used to examine the untranslated 3'-region of the neural PTH gene. Total RNA (5 µg) was denatured for 10 min at 92°C and then reverse transcribed with 200 U Moloney murine leukemia virus reverse transcriptase in the presence of 4 µl 5 × RT buffer, 1.3 µl dNTPs (from 15-mM stocks of each dNTP), 0.25 µl (10 U) RNasin, and 0.5 µl Q<sub>o</sub> primer (5'-GACTCGAGTCGACATCGTTTTTTTTTTTTTTT-3'; 100 ng/µl). After 2 h at 37°C, the reaction was terminated by dilution to 1 ml with TE (10 mM Tris-HCl, pH 7.5) and 1 mM EDTA, and the cDNA was stored at 4°C until use.

The cDNA was subsequently amplified by seminested PCR, as a single round of PCR resulted in the production of numerous nonspecific fragments (data not shown). First, an aliquot of the cDNA (1/200th of the total reaction) was amplified in the presence of oligonucleotide primers MTN2 and Q<sub>o</sub> (5'-GACTCGAGTCGACATCG-3'; 25 pmol of each), dNTPs (1.5 mM of each), 1 × Taq polymerase buffer (Promega), and 10% dimethylsulfoxide. Taq polymerase (2.5 U; Promega) was added after an initial 5-min denaturation, as described by Frohman (14). The mixture was subsequently overlaid with 30 µl mineral oil (Life Brand, Shoppers Drug Mart, Edmonton, Canada) and subjected to initial annealing (52°C, 2 min) and extension (72°C, 40 min) steps before 35 amplification cycles (94°C, 1 min; 52–60°C, 1 min; and 72°C, 3 min) and a final extension (72°C, 15 min). An aliquot (1 µl) of the amplification reaction was diluted in TE (1:20) and amplified under identical conditions in the presence of Q<sub>o</sub> and an internal primer, MTN1.

3'-RACE products and FX174 RF DNA/*HinfI* (Promega) markers were then subjected to electrophoresis through 1.5% agarose gels, stained with ethidium bromide, and viewed under UV light.

### Southern blot analysis

Sequence homology between hypothalamic and parathyroidal PTH cDNAs was further assessed by Southern blotting, using a complementary <sup>32</sup>P-radiolabeled PTH cDNA probe (corresponding to the cDNA region flanked by primer set MTN1). After electrophoresis in 1.5% (wt/vol) agarose, ethidium bromide-stained gels, cDNA moieties were transferred by capillarity to nylon membranes, where they were rinsed in 6 × SSC (1 × SSC = 0.15 mol NaCl/liter and 0.015 mol sodium citrate/liter, pH 7.2) and baked at 80 °C for 2 h. The membranes were prehybridized for 2 h at 42 °C in 30% (vol/vol) formamide containing 6 × SSC, 5 × Denhardt's solution [0.1% (wt/vol) Ficoll, 0.1% (wt/vol) BSA, 0.2% (wt/vol) sodium dodecyl sulfate (SDS), and 0.1% (wt/vol) polyvinylpyrrolidone], 10% (wt/vol) dextran sulfate, and salmon sperm DNA (50 µg/ml; Sigma Chemical Co., St. Louis, MO). The membranes were then hybridized under the same conditions for 18 h in the presence of a [ $\alpha$ -<sup>32</sup>P]dCTP-labeled (200 µCi/mmol; New England Nuclear, Mississauga, Canada) random primer (BRL)-generated (10) cDNA probe, homologous to the region of the PTH gene spanned by the primer set MTN1 and MTN. The probe was generated by cloning a PTH RT-cDNA PCR product into a TA vector (pCR3), as described below. Once this plasmid was sequenced, the cDNA region spanned by the MTN1/MTN fragment was cut from the plasmid with *EcoRI* and electrophoresed in 1.5% (wt/vol) agarose ethidium bromide-stained minigels (10). The cDNA probe was excised from the gel and purified from agarose using Gene Clean II (BioCan Scientific). The cDNA probe was then resuspended in double distilled water before random primer labeling. After a brief rinse in 2 × SSC, the nylon membranes were washed at room temperature (15 min) in 0.1% (wt/vol) SDS containing 2 × SSC and subsequently washed twice (15 min each) at 65 °C in 1% (wt/vol) SDS containing 0.1 × SSC. Blots were then exposed to Kodak (X-Omat AR) x-ray film (Eastman Kodak, Rochester, NY) for 20–40 min.

### Nucleotide cloning and sequencing

The identity of the hypothalamic PTH cDNA was determined by nucleotide sequencing. cDNA moieties were electrophoresed in 1.5% (wt/vol) agarose, ethidium bromide-stained minigels (10). The visualized cDNA bands from PCR (459, 372, and 285 bp) and 3'-RACE (499 bp) were excised from the gel and purified from excess nucleotides and agarose using Gene Clean II (BioCan Scientific), and the fragments were then resuspended in double distilled water before cloning into a TA vector (pCR3), using a Eukaryotic TA Cloning Kit (Invitrogen, San Diego, CA), according to the manufacturer's instructions. Ten colonies were selected for plasmid isolation and restriction analysis (*EcoRI*) for the presence of the insert. Selected colonies were grown overnight in Luria-berthani broth (50 µg/ml kanamycin) before plasmid purification, using a Mini Prep kit (Quiagen, Chatsworth, CA), and resuspended in 60 µl TE buffer. Automated sequencing (Biochemistry Sequencing Lab, University of Alberta, Edmonton, Canada) of plasmids containing PCR and 3'-RACE fragments was performed from both 5' and 3' directions.

### Northern blot analysis

The relative abundance of PTH mRNA in rat neural tissues was determined by Northern blotting. Total RNA from rat hypothalamus (20 µg), extrahypothalamic brain (20 µg), liver (20 µg), and parathyroid glands (0.0625–0.25 ng) was quantified using a fluorimeter and subjected to electrophoresis in a 1% (wt/vol) agarose and 3.1% (wt/vol) formaldehyde gel, stained with ethidium bromide, and transferred to a nylon membrane by capillarity. A 285-bp rPTH complementary RNA (cRNA) probe was constructed from the same plasmid used to produce the cDNA probe for Southern blotting. The plasmid pCR3 (Invitrogen) containing the probe was linearized by *HindIII* digestion and transcribed by Sp6 polymerase, using a riboprobe kit (Promega), in the presence of [ $\alpha$ -<sup>32</sup>P]CTP (800 µCi/mmol; New England Nuclear, Mississauga, Canada). The cRNA probe was then hybridized with the immobilized RNA in 60% (wt/vol) formamide containing 6 × SSC, 0.2% (wt/vol) SDS, and

1 × Denhardt's reagent [0.1% (wt/vol) Ficoll, 0.1% (wt/vol) BSA, 0.1% (wt/vol) polyvinylpyrrolidone, and 100 µg denatured salmon sperm DNA/liter, pH 6.8] for 12 h at 53 °C after a 3-h incubation in the absence of the probe. After a brief rinse in 2 × SSC, the nylon membranes were washed at room temperature (15 min) in 0.1% (wt/vol) SDS containing 2 × SSC and subsequently washed twice (15 min each) at 65 °C in 1% (wt/vol) SDS containing 0.1 × SSC. Membranes were then placed between intensifying screens and exposed to Kodak X-Omat-AR film for 1–7 days. The sizes of the hybridizing RNA moieties were determined by comparison to the 18S and the 28S ribosomal bands visualized on ethidium bromide-stained agarose gels using UV light. Laser densitometry was used to determine the relative abundance of the hybridizing bands. For comparative purposes, similar blots were also probed with a sense cRNA probe generated by transcription of the pCR3 plasmid (Invitrogen) with T7 polymerase.

### Western blot analysis

As cDNA sequences coding for PTH-(1–84) were found in the brain, the presence and relative abundance of this peptide in the hypothalamus was determined by Western blotting. Frozen tissues (hypothalamus and parathyroid) were homogenized (1 g/10 ml) in 1% (wt/vol) SDS, 1 mmol phenylmethylsulfonylfluoride/liter, and 10 µg/ml aprotinin using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged at 2000 × g for 5 min at 4 °C, and 20 µg hypothalamic and 0.1 µg parathyroidal protein [determined by the Bradford method (15)] were diluted 1:1 with loading buffer [0.06 mol Tris-HCl/liter (pH 6.8), (vol/vol) glycerol, 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, and 0.001% bromophenol blue] and heated to 55 °C for 15 min before loading. Proteins were then separated by electrophoresis through a 15% SDS-polyacrylamide gel and transferred electrophoretically (30 V, 4 h, 4 °C) to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding sites on the membrane were blocked (1 h at room temperature) by incubation in 5% nonfat dried milk dissolved in Tris-buffered saline (TBS; 25 mmol Tris-HCl/liter, pH 7.5, and 0.5 mol NaCl/liter). PTH immunoreactivity was detected using a polyclonal guinea pig antibody (16). The antibody, specific for the carboxyl region of bovine (b) PTH-(1–84), was diluted 1:400 in TBS-5% nonfat dried milk and incubated with the membrane for 6 h at room temperature. Membranes were then incubated with a horseradish peroxidase-conjugated antiguinea pig immunoglobulin G (Amersham, Mississauga, Canada) diluted 1:1000 in TBS-5% nonfat dried milk. Membranes were exposed to Kodak X-Omat-AR film, and visualization was achieved using an enhanced chemiluminescence detection system (ECL kit, Amersham). Antibody specificity was determined using bPTH-(1–84) (Peninsula Laboratories, Belmont, CA) as a standard (1 µg) and by preabsorption of the antibody with excess (>10 µg/ml) bPTH-(1–84) and substitution of the antibody with normal (preimmune) serum. Laser densitometry was used to determine the relative abundance of the immunoreactive proteins.

## Results

### PCR amplification

PCR amplification of reverse transcribed hypothalamic RNA in the presence of the oligonucleotide primer MTN and the oligonucleotides MTN1, MTN2, and MTN3 readily generated cDNA moieties of the expected sizes (285, 372, and 459 bp, respectively), identical to those generated from reverse transcribed parathyroidal total RNA (Fig. 2). Similar moieties were generated from reverse transcribed pituitary total RNA and from total RNA and poly(A)<sup>+</sup> mRNA from extrahypothalamic brain and hypothalamic tissues (Fig. 3a). These moieties were not, however, generated when reverse transcribed liver RNA was amplified by 35 cycles of PCR (data not shown) or when hypothalamic RNA or poly(A)<sup>+</sup> mRNA was not transcribed by reverse transcriptase (Figs. 2 and 3a, respectively).



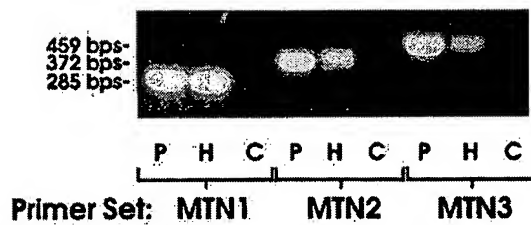


FIG. 2 Analysis of neural PTH transcripts by PCR in the presence of PTH primer sets MTN1/MTN, MTN2/MTN, and MTN3/MTN. RNA from rat parathyroid gland (P) and hypothalamus (H) was reverse transcribed and amplified in the presence of each oligonucleotide primer set. The amplified cDNA was visualized by electrophoresis in ethidium bromide-stained 1.5% minigels, and the size of the fragments was determined by  $\phi$ X174RF DNA/*Hinf*I size markers. Lane C shows hypothalamic RNA in the absence of RT.

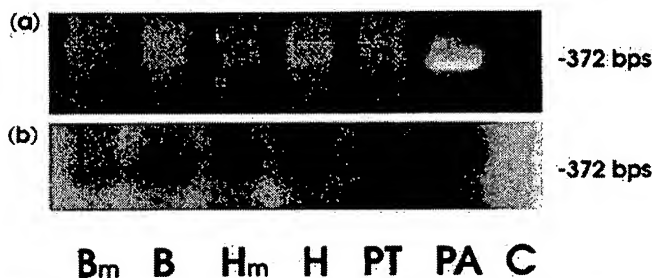


FIG. 3 A, Analysis of neural PTH transcripts by PCR in the presence of PTH primer set MTN2/MTN. Poly(A)<sup>+</sup> mRNA from extrahypothalamic (B<sub>m</sub>) and hypothalamic (H<sub>m</sub>) tissue and total RNA from extrahypothalamic (B), hypothalamic (H), pituitary (PT), and parathyroid (PA) tissue was transcribed and amplified in the presence of oligonucleotide primer set MTN2/MTN. The amplified cDNA was visualized by electrophoresis in 1.5% ethidium bromide-stained minigels, and the sizes of the fragments were determined by  $\phi$ X174 RF DNA/*Hinf*I size markers. Lane C is a negative control containing hypothalamic RNA in the absence of reverse transcriptase. B, Southern analysis of the RT-PCR transcripts (A) with a <sup>32</sup>P-radiolabeled PTH cDNA fragment.

#### Seminested PCR

Reamplification of the 459-bp hypothalamic cDNA (MTN3/MTN) fragment with the nested oligonucleotide primers MTN2/MTN produced a smaller moiety of the predicted size (372 bp), identical to that generated from the corresponding parathyroidal template cDNA (Fig. 4a).

#### Endonuclease digestion

Restriction endonuclease digestion of the 459-bp hypothalamic cDNA (MTN3/MTN) fragment generated smaller moieties of 217 and 242 bp (Fig. 4b). Fragments of identical size were also produced after *Bst*NI digestion of amplified parathyroidal cDNA (Fig. 4b).

#### 3'-RACE

Amplification of reverse transcribed brain tissue RNA with the oligonucleotide primers MTN2 and Q<sub>0</sub> generated a 586-bp moiety (data not shown) identical in size to that generated from reverse transcribed parathyroidal RNA. Multiple weakly staining larger and smaller cDNA moieties were also generated. In the absence of RT, a cDNA fragment

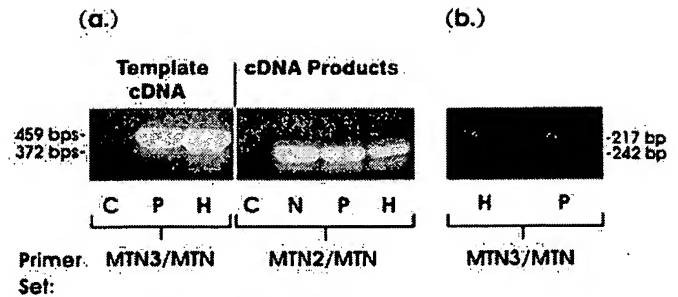


FIG. 4 a, Seminested PCR cDNA fragments generated from parathyroidal (P) and hypothalamic (H) RNA by RT-PCR using primer set MTN3/MTN were excised from the agarose gel and reamplified with the internal primer set MTN2/MTN, generating hypothalamic and parathyroidal cDNA fragments (H and P, respectively). A positive control, using RT cDNA from parathyroidal RNA (N) was also amplified using primer set MTN2/MTN and compared in size to the reamplified fragments. Negative controls (C) of hypothalamic cDNA lacked RT. b, *Bst*NI digestion of hypothalamic (H) and parathyroidal (P) MTN3/MTN cDNA fragments. The amplified cDNA was visualized by electrophoresis in 1.5% ethidium bromide-stained minigels, and the sizes of the fragments were determined by  $\phi$ X174RF DNA/*Hinf*I size markers.

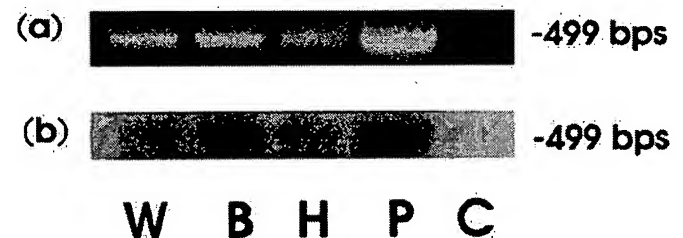


FIG. 5 (a), Analysis of 3'-neural PTH transcripts by 3'-RACE in the presence of primer set MTN2 and Q<sub>0</sub> followed by a second round of 3'-RACE amplification with internal primer set MTN2 and Q<sub>0</sub>. RNA from rat whole brain (W), extrahypothalamic brain (B), hypothalamus (H), and parathyroid gland (P) was reverse transcribed with oligonucleotide primer Q<sub>0</sub> and amplified with two subsequent RACE oligonucleotide primer sets. The amplified cDNA was visualized by electrophoresis in 1.5% ethidium bromide-stained minigels, and the sizes of the fragments were determined by  $\phi$ X174RF DNA/*Hinf*I size markers. Hypothalamic RNA in the absence of RT acted as negative control (C). (b), Southern analysis of the 3'-RACE transcripts (a) with a <sup>32</sup>P-radiolabeled cDNA fragment.

could not be generated from extrahypothalamic RNA (data not shown).

Under similar conditions, reamplification of the RACE mixture from primer set MTN2/Q<sub>0</sub> with the oligonucleotide primer MTN1 and Q<sub>0</sub> generated a cDNA moiety of 499 bp, identical to that generated from RACE amplification of the corresponding parathyroidal template cDNA (Fig. 5a).

#### Southern blot analysis

Hybridization to the <sup>32</sup>P-radiolabeled 285-bp hypothalamic cDNA (MTN1/MTN) probe was observed with the 372-bp cDNA PCR fragments derived from reverse transcribed pituitary, hypothalamic, and extrahypothalamic brain total RNA and from hypothalamic and extrahypothalamic brain poly(A)<sup>+</sup> mRNA. Hybridization to the 372-bp parathyroidal cDNA was similarly observed (Fig. 3b). Hybridization of the probe to the 499-bp cDNA fragments gen-

(36)  
 AGTCCAGTTC ATCAGCTGTC TGGCTTACTC CAGCTTAATA CAGGGTCACT  
 CCTGAAGGAT CCTCTCTGAG AGTCATTGTA TGTGAAGATG ATGTCTGCAA  
 GCACCATGGC TAAGGTGATG ATCTCATGTC TGGCAGTTTG TCTCCTTACC  
 CAGGCAGATG GGAAACCCGT TAAGAAGAGA GCTGTCTAGTG AAATACAGCT  
TATGCACAAC CTGGGCAAAAC ACCTGGCCCTC TGTGGAGAGG ATGCAATGGC  
TGAGAAAAAA GCTGCAAGAT GTACACAATT TTGTTAGTCT TGGAGTCCAA  
ATGGCTGCCA GAGAAGGCAG TTACCAGAGG CCCACCAAGA AGGAGGAAAA  
TGTCCTTGTT GATGGCAATT CAAAAAGTCT TGGCGAGGGG GACAAAGCTG  
ATGTGGATGT ATTAGTTAAG GCTAAATCTC AGTAAATGCT GACGTATTCT  
 AGACCGTGCT GAGCAATAAC ATATGCTGCT ATCCTTTCAA GCTCCACGAA  
 GATCAACAGT GCTAATTCCT CTACTGTAAT AAAAGTTTGA AATTGATTC  
 CACTTTTGTCT CTTAAGGTC TCTTCCAATG ATTCCATTTC AATATATTCT  
 TCTTTTAAA GTATTACACA TTCCACTTC TCTCCTTAAA TATAAATAAA  
 (709)  
 GTTAAATGAT CATGAACCAA A

FIG. 6 Automated nucleotide sequencing of the cloned PCR and 3'-RACE products between cDNA residues 36–709 (corresponding to nucleotides 36–2293 of the PTH gene) (12, 13) were identical (100% homology) to those of parathyroidal PTH cDNA. The homology of these sequenced 673 nucleotides extended over 87 bp of the 5'-noncoding region, throughout all of the coding region (345 bp), and through 239 bp of the 3'-noncoding region. The nucleotide sequence coding for PTH-(1–84) is underlined.

erated by 3'-RACE amplification of the same samples was also demonstrated (Fig. 5b). Hybridization was not observed in the negative control.

#### Nucleotide cloning and sequencing

The PCR and 3'-RACE fragments sequenced encompassed 95% of the parathyroidal PTH cDNA (13) between residues 36–704, corresponding to nucleotides 36–2293 of the PTH gene. The nucleotide sequences of these products were identical (100% homology) to those of parathyroidal PTH cDNA. This homology extended over 87 bp of the 5'-noncoding region, through all of the coding region (345 bp), and through 239 bp of the 3' noncoding region (Fig. 6).

#### Northern blot analysis

Hybridization of the  $^{32}\text{P}$ -radiolabeled cRNA probe occurred to a hypothalamic RNA moiety of approximately 800 bp, identical in size to a hybridizing moiety in parathyroidal RNA (Fig. 7). The relative abundance of this hypothalamic moiety was, however, less than 0.01% of that in the parathyroid gland. Hybridization to a moiety of similar size was also detected in RNA derived from extrahypothalamic brain tissue (data not shown). This probe did not, however, hybridize to liver RNA (data not shown). Moreover, in contrast with the antisense probe, the PTH sense cRNA probe did not hybridize to hypothalamic or parathyroidal RNA (data not shown).



FIG. 7 Northern analysis of PTH mRNA in parathyroidal and extrapathal tissues. Total cellular RNA extracted from hypothalamic (H), extrahypothalamic brain (B), liver (L), and parathyroidal (P) tissues was subjected to electrophoresis and immobilized on nylon membranes. PTH mRNA-like species were visualized by hybridization with a [ $^{32}\text{P}$ ]CTP-labeled rPTH cDNA probe and autoradiography. The position of the 18S ribosomal band is indicated.

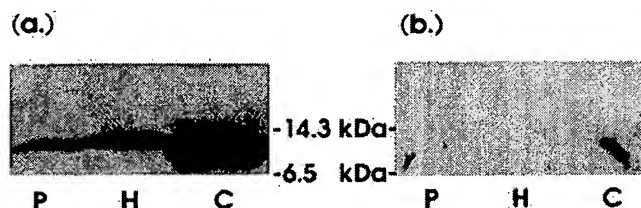


FIG. 8 Immunoblot detection of PTH in rat neural tissue. Crude tissue homogenates (P, parathyroid gland; H, hypothalamus) and bPTH-(1–84) (C) were subjected to reducing SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and incubated with bPTH antiserum (abPTH; A). Identical blots were also incubated with abPTH preabsorbed with excess bPTH (B). Immunoreactive proteins were visualized using horseradish peroxidase-labeled anti-guinea pig immunoglobulin G and enhanced chemiluminescence.

#### Western blot analysis

A single hypothalamic protein of approximately 10 kDa cross-reacted with antibodies directed against bPTH-(1–84) (Fig. 8), identical in size to that detected in the parathyroid gland and comparable in size to bPTH-(1–84). These proteins were not visualized when the primary antibody was replaced by preimmune serum (data not shown) or when it was preabsorbed with excess bPTH-(1–84). The relative abundance of the immunoreactive protein in the hypothalamus was approximately 0.25% of that in the parathyroid gland.

#### Discussion

These results clearly demonstrate the presence of PTH cDNA, coding for PTH-(1–84), within the brain. The brain is thus likely to be an extrapathal site of PTH gene expression, especially as neural PTH cDNA is at least 95% homologous to parathyroidal PTH cDNA and is of comparable size and antigenicity to parathyroidal PTH-(1–84). The PTH gene in the brain is, therefore, likely to be the same as that expressed in the parathyroid gland, rather than a closely related gene. The neural PTH gene has, however, yet to be fully cloned, and the possibility that it may differ from the parathyroidal PTH sequence in the 5'-untranslated region remains.

The results of this study confirm and extend the preliminary findings reported by Fraser *et al.* (7) and demonstrate the presence of a single PTH mRNA in hypothalamic and extrahypothalamic brain tissue identical in size to parathyroidal mRNA. In contrast, Rubin *et al.* (17) reported that human placental cells express a PTH-like mRNA that codes for a protein with homology to the carboxy-terminal residues of PTH, but this transcript was found to be 200–250 nucleotides smaller than human parathyroid adenoma PTH mRNA.

In recent studies, ectopic transcription of the PTH gene has also been demonstrated in hepatocytes, lymphocytes, and lymphoblastoid cells (18). However, as the detection of PTH gene transcripts in these tissues was dependent upon booster PCR, Handt *et al.* (18) considered this reflected the phenomenon of illegitimate transcription, especially as the abundance of these transcripts was not regulated by vitamin D<sub>3</sub> or phorbol esters. The occurrence of PTH gene transcripts in the brain is, however, unlikely to be due to illegitimate transcription (8). Although these transcripts were only detected by Fraser *et al.* (7) after booster PCR, cDNA fragments were not similarly generated when reverse transcribed liver RNA was amplified by two cycles of PCR under identical conditions (7). Indeed, we were similarly unable to generate any PCR fragments from reverse transcribed liver RNA in the present study, even after 45 cycles of PCR (data not shown). Moreover, when the oligonucleotide primers used by Fraser *et al.* (7) were subsequently used in the present study without the *EcoRI/HindIII* linker sequences, cDNA moieties of predicted size and sequence were readily generated after a single round (35 cycles) of PCR. Expression of the PTH gene in the brain, unlike that in hepatocytes and immune cells (18), would thus appear to be physiological, rather than a methodological artifact, even though PTH mRNA is a rare neural transcript.

The abundance of PTH mRNA in the hypothalamus was far less than that in the parathyroid gland, although the level of PTH gene expression in discrete nuclei was not determined. Indeed, Fraser *et al.* (7) were only able to localize, by *in situ* hybridization, PTH mRNA in the supraoptic and paraventricular nuclei of the rat hypothalamus. The transcription of the PTH gene in the brain may, therefore, be confined to a relatively small number of neural cells. These cells may not, however, be confined to the hypothalamus, as PTH mRNA was also detected in the present study in extrahypothalamic brain tissue. This finding contrasts with the *in situ* hybridization data of Fraser *et al.* (7), but is consistent with the widespread distribution of PTH immunoreactivity in the mammalian brain (2, 6).

The presence of PTH immunoreactivity in the brain suggests translation of the neural PTH message. This immunoreactivity is primarily associated with a protein identical in size to PTH-(1–84), as demonstrated by Western blotting and HPLC fractionation (1). Thus, although PTH-degrading enzymes are present in neural tissues (19), the relative rate of PTH degradation into amino- and carboxy-terminal fragments may be far less than that in peripheral tissues. Moreover, as the relative abundance of brain PTH (~0.25% of that in the parathyroid gland) was greater than the relative abundance of brain PTH mRNA (~0.01%), the relative rate of PTH

degradation in the brain may be less than that in the parathyroid glands. The rate of PTH mRNA turnover may, alternatively, differ in hypothalamic tissue.

The low abundance of PTH immunoreactivity in the brain suggests that it is unlikely to contribute to the pool of PTH in the systemic circulation, especially as some researchers (4, 5, 20) consider the blood-brain barrier to be impermeable to PTH. The permeability of this barrier is, however, uncertain. Indeed, the PTH concentration in cerebrospinal fluid (5, 20) is higher than that likely to result from local production (20–70% of the plasma concentration), and in some studies, cerebrospinal fluid PTH levels correlate with plasma PTH concentrations (21–23). Therefore, the possibility that PTH of neural origin may, conversely, enter the systemic circulation exists, especially as the implantation of parathyroid glands in the brains of parathyroidectomized rats is able to maintain peripheral plasma PTH levels within the normal range (24). It is, thus, of interest that immunoreactive PTH (albeit at very low concentrations) has frequently been measured in the peripheral plasma of hypoparathyroid and parathyroidectomized patients (25–27) and is measurable in the peripheral plasma of fish and amphibians that lack parathyroid glands, but have PTH immunoreactivity in their central nervous system (1, 28). Under physiological conditions it is, however, unlikely that neural PTH would be present in the peripheral plasma of mammals at concentrations sufficient to induce biological effects in the traditional target sites of parathyroidal PTH (e.g. renal and osseous tissue). It is, therefore, possible that PTH produced within the brain has autocrine or paracrine actions to modulate neural function.

It is now well established that PTH has neurological actions (see Ref. 29 for review), modulating Ca<sup>2+</sup> flux (30, 31), phosphoinositol metabolism (32), and the synthesis, release, and metabolism of neurotransmitters (33–35). Indeed, the actions of PTH within the brain may induce neurological dysfunction (29) and affect central behavior (36, 37). A central action of PTH within the rat brain has also recently been considered to participate in the regulation of peripheral Ca<sup>2+</sup> homeostasis (38). Intracerebroventricular injections of PTH have been shown to inhibit neuronal activity in the ventromedial nucleus of the hypothalamus and inhibit urethane-induced hypocalcemia (38).

The neural actions of PTH are likely to be receptor mediated, especially as membrane-binding sites for PTH and/or PTH-related protein (PTHrP) have been found in the cerebrum, the cerebellum, and, particularly, the hypothalamus (39). The demonstration of PTH/PTHrP receptor mRNA in the rat cerebral cortex and cerebellum (40) also suggests that the brain is a target site for PTH action.

Within the brain, PTH would appear to affect both neuronal and glial cells. PTH *in vitro* has direct effects on isolated neurons (31, 41) and in brain cell cultures that do not contain neurons (42, 43), in which PTH/PTHrP receptor mRNA is expressed in astrocytes (43). These neural receptors may, however, normally mediate actions of PTHrP rather than PTH, as its expression in neural tissue is greater than the expression of PTH (44, 45). The recent discovery of PTH-specific (PTH2) receptors within the brain (46) nevertheless suggests that PTH has physiological roles in neural regulation. Indeed, PTH2 receptors are

more abundant in brain than in kidney or osseous tissues (46), suggesting that the brain is a major target site for PTH action. Furthermore, these PTH-specific receptors may be responsible for the demonstrated effects of PTH on the firing of ventromedial nucleus neurons (38), as neither PTHrP nor PTH/PTHrP receptor mRNA is present within this nucleus (47). Similarly, although PTH/PTHrP receptor mRNA levels are relatively low in the paraventricular and supraoptic nuclei (47), these are areas of the rat brain in which PTH mRNA is most clearly demonstrated (7). These results, therefore, suggest that PTH may have autocrine or paracrine effects within the brain, particularly within the hypothalamus.

In summary, these results demonstrate the presence of PTH cDNA, coding for PTH-(1–84), within neural tissues and suggest local roles for PTH within the central nervous system.

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# EXHIBIT 3

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# Parathyroid Hormone Messenger Ribonucleic Acid in the Rat Hypothalamus\*

R. A. FRASER, H. M. KRONENBERG, P. K. T. PANG, AND S. HARVEY

Department of Physiology (R.A.F., P.K.T.P., S.H.), University of Alberta, Edmonton, T6G 2H7, Canada;  
Department of Medicine (H.M.K.), Massachusetts General Hospital, Harvard Medical School, Boston,  
Massachusetts, 02114

**ABSTRACT.** Polyadenylated RNA, extracted from rat hypothalami, cross-hybridized with a RNA probe complementary in sequence to rat PTH (rPTH) messenger RNA (mRNA). Amplification of complementary DNA (cDNA) by the polymerase chain reaction also demonstrated the presence of rPTH mRNA in the rat hypothalamus and parathyroid gland. rPTH mRNA

was localized by *in situ* hybridization in the paraventricular and supraoptic nuclei of the rat hypothalamus. These results demonstrate the expression of the PTH gene in the central nervous system of the rat in areas which suggest roles for PTH in neuroendocrine function. (*Endocrinology* 127: 2517-2522, 1990)

**E**CTOPIC hormone production has been demonstrated by the diverse distribution of immunoreactive (IR) polypeptide hormones in endocrine and nonendocrine tissues (1-4). PTH, the major peptide regulator of blood calcium is synthesized primarily in the parathyroid gland. IR PTH however has also been found in human cerebrospinal fluid (5) and in the brain and pituitary gland of sheep (6, 7). More recently, heat-stable, nondialyzable IR PTH-like activity has been localized in the brain and pituitary gland of piscine (8), amphibian, reptilian, avian, and mammalian species (9, 10). This IR PTH has been shown to coelute with authentic PTH from reverse-phase Sep Pak C<sub>18</sub> preparative and HPLC columns (9). Immunocytochemical studies have also indicated that peptides with IR PTH are confined to perikarya in specific hypothalamic nuclei (10). The possibility that the PTH gene may, therefore, be expressed in the brain has been determined in the present study, using a specific complementary RNA probe to PTH messenger RNA (mRNA).

## Materials and Methods

### RNA preparation

Total RNA was prepared following a modified procedure of Okayama *et al.* (11). Briefly, hypothalami, liver, and parathy-

roid glands were dissected from 250 g male Sprague-Dawley rats (Harlan Co., Indianapolis, IN) and immediately frozen in liquid nitrogen. Approximately 1-2 g of the liver and hypothalamii and 200  $\mu$ g of the parathyroidal frozen tissue, in 5.5 M guanidinium thiocyanate solution (1:10 wt/vol), were homogenized by a polytron (Brinkman Instruments, Westbury, NY) and centrifuged at  $1,500 \times g$  at 22 C for 5 min. The supernatants were passed through an 18 g needle to shear DNA (12) and centrifuged at  $5,000 \times g$  at 15 C for 20 min. The supernatants were then subjected to isopycnic ultracentrifugation ( $125,000 \times g$  at 15 C for 24 h) through a cesium trifluoroacetic acid bed (density  $1.51 \pm 0.01$  g/ml; Pharmacia Fine Chemicals, Uppsala, Sweden), containing 0.1 M EDTA, pH 7.0. The RNA pellets were resuspended in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA buffer (TE) (12), heated to 65 C and centrifuged to remove insoluble material. The yield of RNA was  $0.094 \pm 0.01\%$  of total tissue wt, as determined by spectral analysis at 260 nm, and its purity was assessed by ethidium bromide staining after 1% agarose minigel electrophoresis (12).

Polyadenylated (poly A<sup>+</sup>) RNA was separated from total hypothalamic RNA using oligo-deoxythymine cellulose spin columns (Pharmacia Fine Chemicals) and precipitated with 10 M ammonium acetate (0.2 vol/vol) and ethanol (3 vol/vol) at -80 C for 1 h. The poly A<sup>+</sup> RNA pellet was dried and resuspended in diethylpyrocarbonate (DEPC) treated water before spectral quantification and minigel analysis.

### RNA probe synthesis

A portion of the rPTH gene (13) containing all of exon III and intron B was subcloned into a pGEM4 vector (Promega Corporation, Madison, WI) by Dr. Gerhard Heinrich (Fig. 1a). A second plasmid (Fig. 1b) was constructed by inserting the *Bam*HI/*Hind*III chicken PTH (cPTH) (14) fragment into pGEM2 vector (Promega Corp.). The complimentary, or anti-

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Address requests for reprints to: Dr. S. Harvey, Department of Physiology, Faculty of Medicine/University of Alberta, 7-55 Medical Sciences Building, Edmonton, T6G 2H7 Canada.

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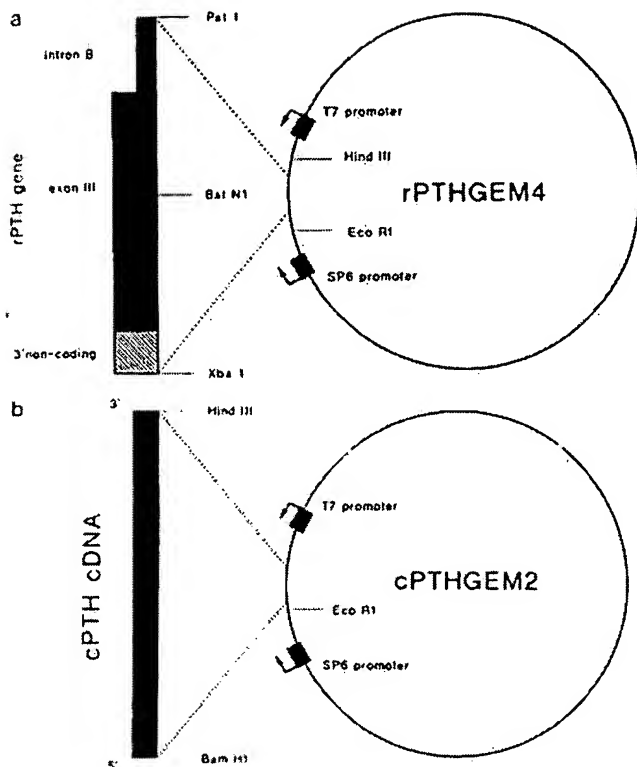


FIG. 1. Plasmid constructs used in RNA probe synthesis. a, rPTHGEM4 containing exon III, intron B, and part of exon II of the rPTH gene used for the synthesis of a 375 bp antisense RNA probe, after *Hind*III digestion; b, cPTHGEM2 containing the cPTH cDNA sequence used for the synthesis of a 430 bp sense RNA probe, after digestion with *Eco*RI.

sense, sequence of rPTH mRNA and the sense sequence of cPTH mRNA were transcribed *in vitro* using *Hind*III-digested (Boehringer Mannheim, Dorval, Quebec, Canada) rPTHGEM4 or cPTHGEM2 as templates and SP6 polymerase (Bethesda Research Laboratories, Bethesda, MD) in the presence of 25  $\mu$ Ci [ $^{32}$ P]cytosine triphosphate (3,000 Ci/mmol) for probing Northern blots and [ $^{35}$ S]cytosine triphosphate (800 Ci/mmol; New England Nuclear Mississauga, Ontario, Canada) for probing *in situ* hybridization, following a modified procedure of Melton *et al.* (15). The sense sequence of cPTH mRNA was used to construct a nonspecific probe since it was of similar size (430 base pairs) as the rPTH gene fragment (375 bp) and could be similarly synthesized. rPTH mRNA was not used to construct a sense riboprobe in view of the possibility that small amounts of the antisense sequence could be simultaneously transcribed *in vivo* (16). The probes were purified from unincorporated RNAs by three consecutive 10 M ammonium acetate (0.2 vol/vol), isopropanol (3 vol/vol) precipitations and resuspended in DEPC-treated water.

#### Northern blot analysis

Total RNA from rat parathyroid gland (1 mg) and poly A<sup>+</sup> RNA from rat hypothalamus (10 mg), in 50% formamide, 0.1% formaldehyde, and 1  $\times$  MOPS (20 mM 3(N-morpholino)propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA,

pH 7.0) (12) were electrophoresed through a 1.2% agarose and 3.1% formaldehyde gel containing 1  $\times$  MOPS. The RNA was transferred by capillarity to nitrocellulose, which was then rinsed in 6  $\times$  SSC (1  $\times$  SSC = 0.15 M NaCl-0.015 M sodium citrate, pH 7.2) and baked at 80 C for 2 h under a vacuum.

The Northern blots were prehybridized for at least 2 h at 65 C in 50% formamide containing 5  $\times$  PIPES (0.75 M NaCl, 25 mM PIPES, 25 mM EDTA, pH 6.8), 5  $\times$  Denhardt's (0.1% Ficoll, 0.1% BSA, 0.2% sodium dodecyl sulfate, and 0.1% polyvinylpyrrolidone), salmon sperm DNA (100 mg/ml), and yeast transfer RNA (tRNA) (100 mg/ml, Sigma Chemical Co., St. Louis, MO) and then hybridized under the same conditions, for 12 h in the presence of the newly synthesized RNA probes. The blots were then serially washed at room temperature and twice at 68 C in 0.2% SDS, containing 2, 0.5, and 0.05  $\times$  SSC, respectively, before exposure to x-ray film (X-OMAT AR, Kodak, Rochester, NY) for periods of 2 h to 1 week.

#### Polymerase chain reaction (PCR)

Rat parathyroid gland total and hypothalamic poly A<sup>+</sup> RNA was reverse transcribed by ribonuclease H<sup>-</sup> Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (100 U, Bethesda Research Laboratories, Gaithersburg, MD) in the presence of 3'-oligomer rPTH antisense primer (50 pmol, Fig. 2b); deoxynucleotides (1.25 mM of each, Boehringer Mannheim, Dorval, Quebec, Canada), and 1  $\times$  PCR buffer [50 mM KCl, 10 mM Tris-Cl, pH 8.4, 1.5 mM MgCl<sub>2</sub>, and 20  $\mu$ g/ml gelatin (17)]. The reactions were diluted with DEPC-treated water (500:1, vol/vol), and an aliquot of each (1/1000 of total vol) was used in a PCR (17) mixture containing both 5'-oligomer rPTH sense and 3'-oligomer rPTH antisense primers (15 pmol of each, Fig. 2), deoxynucleotides (1.25 mM of each), 1  $\times$  PCR reaction buffer, and *Thermus aquaticus* (Taq) DNA polymerase (5 U, Boehringer Mannheim). The mixture was overlaid with mineral oil (vol/vol), heated to 94 C for 2 min before 30 cycles of 65 C annealing for 1 min, 72 C extension for 30 sec, and 94 C denaturing for 30 sec in a thermal reactor (Tyler Instruments, Edmonton, Alberta, Canada). Rat hypothalamic PCR reaction product (0.04 vol) was reamplified under the identical conditions. As a negative control, liver poly A<sup>+</sup> RNA was similarly reverse transcribed and subjected to PCR, as described above.

#### In situ hybridization

Sodium pentobarbital-anesthetized Sprague-Dawley rats were perfused with PBS containing EGTA (100  $\mu$ g/liter) and then

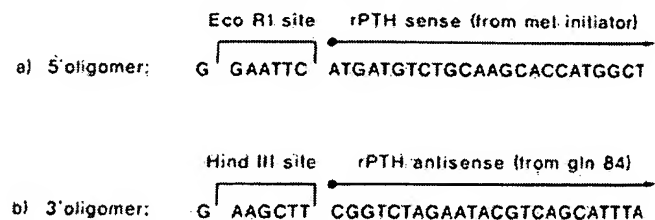


FIG. 2. Oligonucleotide primers used in the reverse transcription and PCR. The 5'-oligomer primer (a) is equivalent to the 5'-end of the mRNA sequence of rPTH, while the 3'-oligomer primer (b) is complementary to the 3'-end of the mRNA sequence.

with 4% paraformaldehyde in phosphate buffer (pH 7.0). Whole brains were dissected and postfixed in 4% paraformaldehyde at 4°C overnight and cryoprotected by sequential saturation in 10, 15, and 20% sucrose-phosphate buffer. Coronal sections, 10  $\mu$ m, were cut using a cryostat (Reichert-Jung, Cambridge Ins. CombH, Heidelberg, West Germany) and mounted onto gelatin (0.4%) chromium potassium sulfate (0.04%)-coated slides.

Tissue sections were fixed with 4% formaldehyde in PBS, perforated with 50 mM Tris-Cl (pH 7.6) and 5 mM EDTA containing proteinase K (20  $\mu$ g/ml, Boehringer Mannheim), and dried with ethanol, before prehybridization in hybridization buffer (50% formamide, 5  $\times$  PIPES, 5  $\times$  Denhardt's, 0.2% SDS, 100 mM dithiothreitol, and 250  $\mu$ g/ml of salmon sperm DNA and yeast tRNA) in a humidified chamber at 42°C for 2 h (18). Alternate sections were hybridized at 42°C for 12 h in hybridization buffer containing either rPTH antisense or cPTH sense RNA probes. Slides were then serially washed at room temperature (21°C) in 4  $\times$  SSC, initially in the presence of 10 mM  $\beta$ -mercaptoethanol. The nonhybridized RNA probes were then digested at 37°C with 50  $\mu$ g/ml ribonuclease A (Boehringer Mannheim) in 0.5 M NaCl in TE, followed by a 2  $\times$  SSC wash at room temperature and finally a 0.1  $\times$  SSC wash at 42°C. Air-dried slides were dipped in autoradiographic emulsion (NTP-2, Kodak, Rochester, NY) and exposed 14 days before developing.

## Results

### Northern blot analysis

As expected, potent hybridization of the rPTH antisense riboprobe with RNA extracted from the parathyroid gland was observed within 2 h of exposure (lane 1, Fig. 3). After 7 days, weak hybridization with hypothalamic poly A<sup>+</sup> RNA was observed with a moiety that comigrated with the signal detected in the parathyroid gland (lane 2, Fig. 3). Under the same conditions and using equivalent amounts of the riboprobe, specific hybridization with liver RNA could not be detected (data not shown), although nonspecific hybridization with the 28S and 18S bands (located on the ethidium bromide-stained gel; data not shown) was evident. Nonspecific

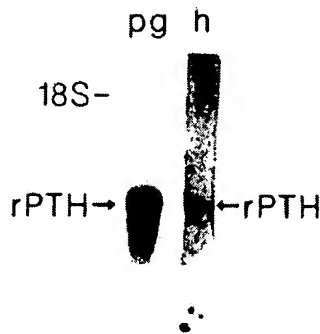


FIG. 3. Northern blot analysis of total RNA extracted from rat parathyroid gland (lane 1) and poly A<sup>+</sup> RNA from rat hypothalamus (lane 2), probed with antisense rPTH RNA probe. The migration of 18S RNA as viewed on ethidium bromide-stained gels, is indicated.

hybridization with these bands was also indicated with the extract of hypothalamic poly A<sup>+</sup> RNA. Identical results were also demonstrated using three further poly A<sup>+</sup> RNA preparations (data not shown).

### PCR

The PCR conducted with parathyroid gland complementary DNA (cDNA) produced a single intense band smaller than the 434 bp marker and larger than the 298 bp marker (lane 1, Fig. 4) as viewed on the ethidium bromide-stained gel (lane 2, Fig. 4). Reamplification of rat hypothalamic cDNA also produced a band of equal size to that of rat parathyroid gland (lane 4, Fig. 4). Amplification of liver cDNA did not reveal a PCR product (lane 3, Fig. 4).

### In situ hybridization

Exposure of the rat brain sections to the emulsion for 14 days indicated specific bilateral hybridization with rPTH riboprobe in the supraoptic (SO) nuclei (Fig. 5, a and b, and Fig. 6). Specific hybridization was also demonstrated in the paraventricular lateral magnocellular (PaLM) nuclei (Figs. 5, d and e and 6). No hybridization with an equivalent amount of the nonspecific cPTH riboprobe was observed in adjacent sections of these or other hypothalamic nuclei (Fig. 5, c and f). Specific hybridization could not clearly be detected in sections exposed to the emulsion for less than 7 days, further indicating the low abundance of the message.

Under higher magnification, clustering of the rPTH signal was found in specific cells of the SO (Fig. 5b). The



FIG. 4. Ethidium bromide-stained gel of the *Hae*III digested pUC 18 low mol wt markers (587, 456, 434, 298, 267, and 174, lane 1) and PCR-amplified cDNA from parathyroidal (lane 2), liver (lane 3), and hypothalamic (lane 4) tissues.

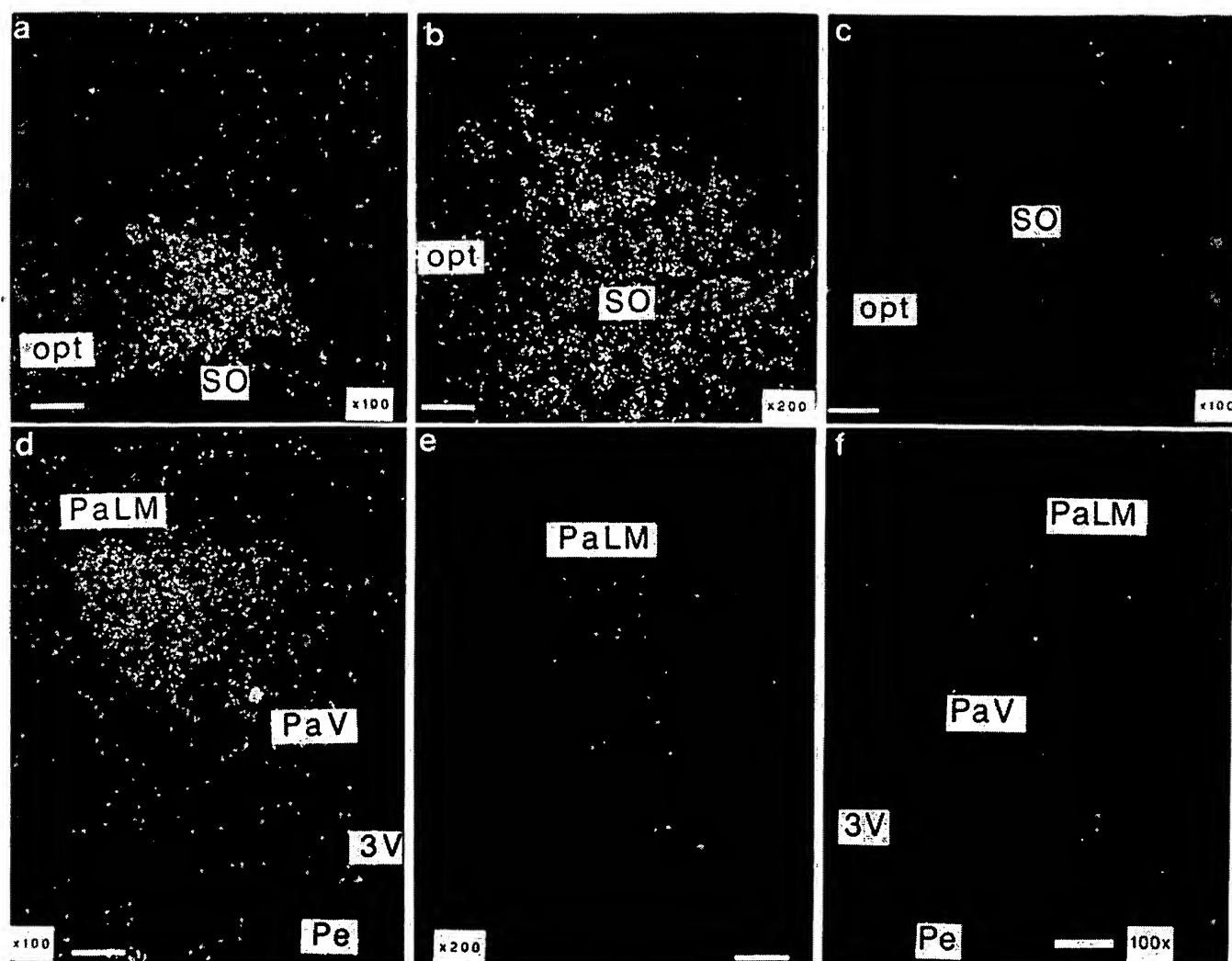


FIG. 5. Dark field microphotographs of the *in situ* hybridization of rat brain cryostat sections with rPTH antisense (a, b, d, and e) and cPTH sense (c and f) RNA probes. Accumulation of silver grains over the cells containing PTH mRNA appear as *white clustering* against the background. Paraventricular lateral magnocellular (PaLM), paraventricular (PaV), periventricular (Pe), and supraoptic nuclei (SO), as well as the third ventricle (3V) and optic tract (opt), are indicated in the micrograph. Bar = 50  $\mu$ m and 25  $\mu$ m for 100 $\times$  and 200 $\times$  magnification, respectively.

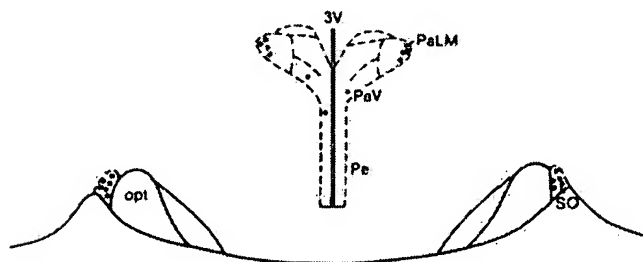


FIG. 6. Schematic drawing of a cross-section through the rat's medial basal hypothalamus indicating the nuclei of highest rPTH mRNA content (●). Abbreviations as in Fig. 5.

tight clustering seen in the SO was also evident in the PaLM, but not the paraventricular nucleus (PaV) or

periventricular nucleus (Pe), although the signal in these nuclei was greater than background (Fig. 5c).

### Discussion

These results demonstrate, for the first time, that a PTH-like mRNA capable of hybridizing with rPTH antisense riboprobe is present in the hypothalamus of rats. Northern blot analysis indicates that this mRNA comigrates with rPTH mRNA, and the absence of other hybridizing bands (except those due to 28S and 18S rRNA) demonstrates the specificity of this signal. Although the signal on the Northern is weak, it was readily detected (under the conditions used) by the increased sensitivity of *in situ* hybridization (19). Moreover, am-

plification of rat hypothalamic cDNA by PCR produced a single band of equal size to parathyroidal PTH cDNA (383 bp; Ref. 14), intense enough to be viewed by ethidium bromide staining. Since the rat hypothalamic cDNA was reverse transcribed from cytoplasmic poly A<sup>+</sup> RNA, the PTH gene would therefore appear to be expressed in the rat hypothalamus, even though the message is of low abundance.

In view of the unique nucleotide sequence of PTH mRNA at the 3'-terminus (13) it is highly unlikely that mRNA other than PTH mRNA could hybridize with the riboprobe, given the stringency of the hybridization conditions used. Although Weir *et al.* (20) reported that a PTH-related peptide (PTHrp) was expressed in rat brain, the sequence homology between PTH and PTHrp is restricted to a short sequence near the 5'-end (21). Since neither RNA probe, synthesized from rPTHGEM4, contains the 5'-region of PTH mRNA and the 5'-primer does not complement PTHrp, it is highly unlikely that the mRNA we detected was an expression of PTHrp gene. Furthermore, whereas we detected PTH mRNA in the hypothalamus Weir *et al.* (20) reported the expression of the PTHrp gene in extrahypothalamic tissues, particularly the cerebral cortex, and cerebral hemispheres. The possibility that the PTH gene may also be expressed in extrahypothalamic brain regions has yet to be examined, although Balabanova *et al.* (7) detected IR PTH throughout the brain.

In the present study cells hybridizing with the PTH mRNA riboprobe were located within the SO and PaLM nuclei of the hypothalamus. In contrast, however, IR PTH was determined by immunocytochemistry primarily within the PaV nuclei and to a lesser extent in the suprachiasmatic, Pe, and SO nuclei of the mouse brain (10). PTH-like immunoreactivity has, nevertheless, also been located in the SO nuclei of other vertebrate species (10, 22). In view of this finding, therefore, it is probable that the IR peptides previously detected in the vertebrate brain resulted from the translation of PTH mRNA expressed in these nuclei. The synthesis or release of these peptides may, however, differ from that in the parathyroid gland, since the content of IR PTH in the rat brain was recently shown to be independent of hyper- and hypocalcemia (9), even though calcium depletion and vitamin D stimulated the release of IR PTH from sheep brain explants *in vitro* (7). The role if any, for brain PTH in peripheral calcium homeostasis or in central function is also uncertain.

The locations of IR PTH and PTH mRNA within the brain are in hypophysiotropic regions of the hypothalamus that may regulate anterior and/or posterior pituitary function. The possibility that brain PTH may function as a hypophysiotropic releasing factor is suggested, since peptidergic IR PTH fibers terminate around hypophysial

blood vessels in the external zone of the mouse median eminence of mammals and in the adenohypophysis of teleosts lacking portal blood vessels (10, 21). The demonstration of increased PRL secretion in mammalian species systemically injected with PTH or parathyroid gland extracts (23-27) may also indicate a neuroendocrine role of this peptide.

In view of the location of PTH within the brain, it is also possible that PTH may participate in neurotransmission. This possibility is supported by the finding that PTH has peripheral actions on target tissues that parallel those induced by  $\beta$ -adrenergic stimulation (28). Actions of PTH on neural tissue (inhibition of Ca<sup>2+</sup> channels in neuroblastoma cells) have also been demonstrated (29), and PTH has also recently been shown to regulate calcium uptake by brain synaptosomes (30, 31). The possibility that PTH has physiological roles within the central nervous system is also indicated by the finding that PTH IR is located in the central nervous system of fish, which lack peripheral parathyroid glands (8).

In summary, these results demonstrate that the PTH gene is transcribed in rat hypothalamic nuclei in regions that suggest roles for PTH in neurotransmission or neuroendocrine function.

### Acknowledgments

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# **EXHIBIT 4**

## Parathyroid Hormone Secretion by Brain and Pituitary of Sheep

S. Balabanova, J. Peter, and G. Reinhardt

Institut für Rechtsmedizin der Universität Ulm

**Summary.** The secretion of immunoreactive PTH by different brain regions and pituitary of sheep was studied in vitro. Separate tissue samples of gyrus, internal capsule, basal ganglia, cerebellum, and pituitary were incubated in culture medium with low, normal, and high Calcium (Ca) (0.7 mM, 1.2 mM and 2.4 mM) concentrations. PTH release in medium containing low Ca were observed by all samples. The concentrations increased in the fifth and sixth hour from 100% (1st hour basis value) up to 300%. The PTH release showed an inverse relationship to the Ca concentration in the culture medium. To induce any significant decrease in medium concentrations of PTH,  $1.25(\text{OH})_2\text{D}$  (100 ng/ml) was added to the culture medium. This effect could be reversed by DB-cAMP. Our results indicate that secretion of immunoreactive PTH by brain and pituitary of sheep may occur in vitro. The secretion depends on the content of Ca,  $1.25(\text{OH})_2\text{D}$ , and DB-cAMP.

**Key words:** Parathormone secretion - CNS - Pituitary

Recently we have shown the presence of immunoreactive PTH in the cerebrospinal fluid and in CNS and pituitary of sheep [5, 6]. We have supposed that this hormone is pituitary or hypothalamic origin. The aim of this study was to examine the secre-

tion of PTH by the CNS and pituitary, and the relationship between the PTH release and Ca,  $1.25(\text{OH})_2\text{D}$ , and DB-cAMP, in vitro.

### Material and Methods

Gyrus (precentral and postcentral), internal capsule, basal ganglia, cerebellum, and pituitary were obtained immediately after killing the animals. Separate tissue samples (0.5 g) of each region, and pituitary (three glands cutted into slices of about 1 mm) were incubated in flasks with 10 ml culture medium (serum-free Eagle's Minimum Essential Medium, Sigma, FRG), with low Ca concentration up to 6 h at 37° C, in an atmosphere of 95% air and 5%  $\text{CO}_2$ . The magnesium concentration was constantly 0.8 mM.

In a parallel incubation, the following experiments were performed:

After 2 h incubation:

1. The medium was altered to a normal and respectively high Ca concentrations and incubated for 2 h.

2. 100 ng/ml  $1.25(\text{OH})_2\text{D}$  were added, and

3. 2 h later 10 mM DB-cAMP were added to the self medium.

The secretion of PTH into the culture medium was determined hourly during a period of 6 h. At the end of each incubation interval, the medium in each flask was centrifuged at 1,000 g for 10 min. The supernatant was decanted and frozen at -20° C. The PTH secretion during the first hour was defined as basis value (100%). The change of the concentration during the following hours was put in reference.

All investigations were performed with three samples of each separate tissue.

**Abbreviations:** ACTH = Adrenocorticotrophic hormone; Ca = Calcium; CNS = Central nervous system; cAMP = Cyclic adenosine 3',5'-monophosphate; CT = Calcitonin; DB-cAMP = Dibutyl- $\gamma$ -cyclic adenosine 3',5'-monophosphate;  $1.25(\text{OH})_2\text{D}$  = Dihydroxyvitamin D; Leu-ENK = Leucine-enkephalin; Mg = Magnesium; Met-ENK = Methionine-enkephalin; PTH = Immunoreactive parathyroid hormone; TSH = Thyroid stimulating hormone;  $\text{T}_4$  = Thyroxine;  $\text{T}_3$  = Triiodothyronine



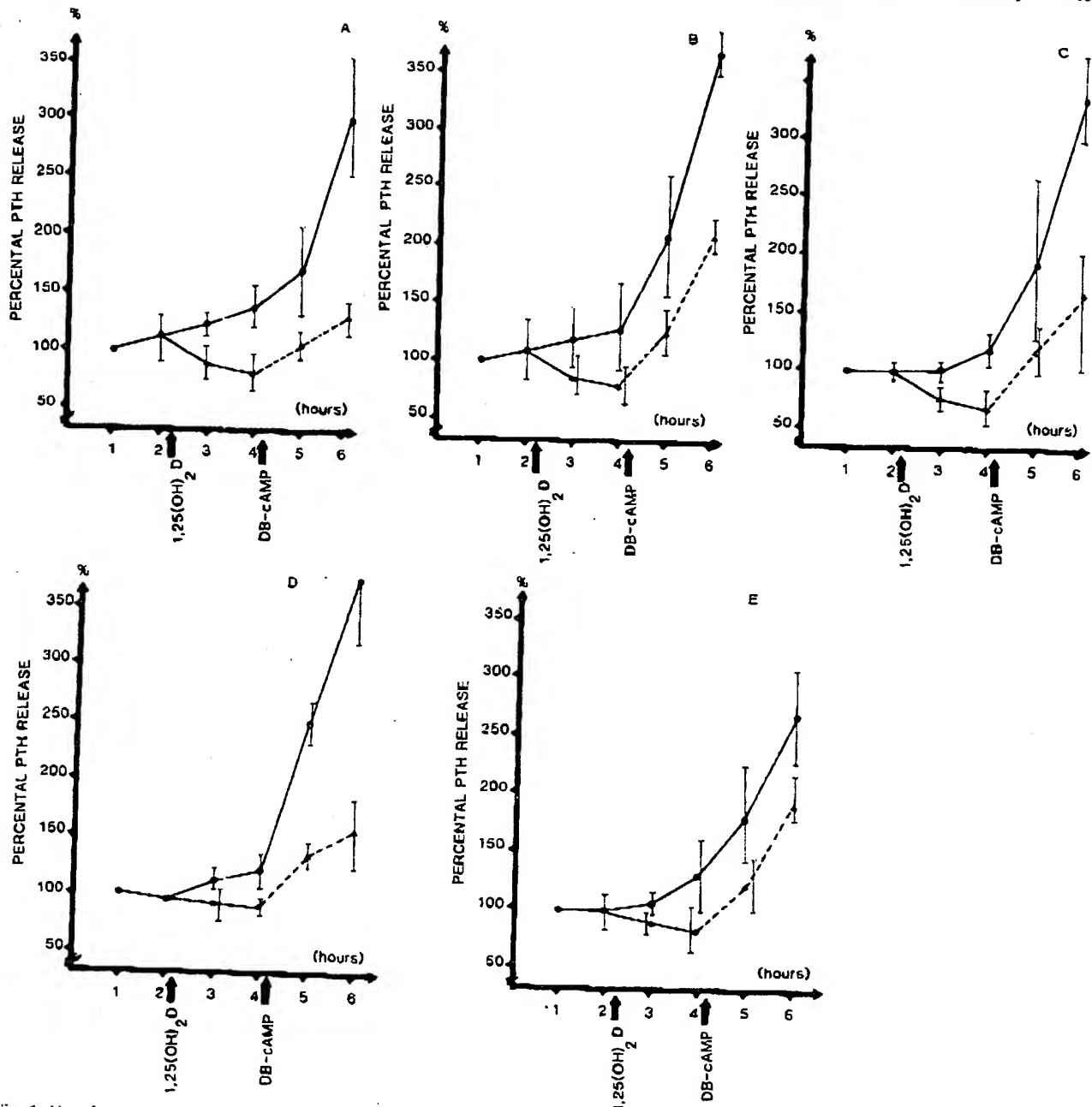


Fig. 1. Hourly PTH release at low Ca concentration (0.7 mM) —●—●—●—, and plus  $1,25(\text{OH})_2\text{D}$  —▲—▲—▲—, and plus  $1,25(\text{OH})_2\text{D}$  + DB-cAMP —■—■—■—■—. Gyrus (A), internal capsule (B), basal ganglia (C), cerebellum (D), and pituitary (E). Each point represents mean  $\pm$  SD. (Results of three incubations for each tissue sample)

The PTH concentrations were measured by radioimmunoassay (35–84 amino acids) (Institut National des Radioelements, Belgium) according to the method of Arnaud et al. [3]. The standard was bovine PTH, the antisera was a rabbit antiserum directed against the C-terminal part (amino acids 35–84) of the molecule. The binding capacity was 30%–35%. The standard curve covers the range 0.3 ng/ml–12.5 ng/ml. The intraassay coefficient of

variation was 7.5%. The fragment 1–34 provoked a reduction of native labelled PTH fixed on antibodies, in a ratio of only 1:10.000 ng/ml. The cross reactions with ACTH, CT, TSH,  $\text{T}_3$ ,  $\text{T}_4$ , Leu-ENK, Met-ENK,  $\alpha$ -endorphine,  $\beta$ -endorphine,  $\gamma$ -endorphine, and  $1,25(\text{OH})_2\text{D}$  were below 0.1%. The nonspecific binding of the tracer to the buffer solution of the assay and to the incubation medium from each culture was under 5%.



Table 1. PTH release in medium with low, high, and normal Ca concentrations. Each value represents mean  $\pm$  SD. (Results of three incubations for each tissue sample)

Tissue	Parathyroid hormone concentrations (ng/ml $\pm$ SD) in culture medium with:					
	Low Ca (0.7 mM)		High Ca (2.4 mM)		Normal Ca (1.2 mM)	
	3 h	4 h	3 h	4 h	3 h	4 h
Gyrus	6.8 $\pm$ 0.7	7.7 $\pm$ 1.0	6.2 $\pm$ 1.2	5.7 $\pm$ 0.8	6.7 $\pm$ 0.9	6.8 $\pm$ 1.0
Internal capsule	5.1 $\pm$ 1.3	5.3 $\pm$ 1.4	4.2 $\pm$ 1.6	4.0 $\pm$ 1.2	4.6 $\pm$ 1.2	4.5 $\pm$ 1.7
Basal ganglia	5.5 $\pm$ 0.4	6.8 $\pm$ 0.3	5.2 $\pm$ 1.3	4.7 $\pm$ 0.3	5.4 $\pm$ 1.8	5.6 $\pm$ 0.5
Cerebellum	6.7 $\pm$ 0.4	7.2 $\pm$ 0.9	4.9 $\pm$ 0.4	4.8 $\pm$ 1.0	5.9 $\pm$ 0.3	6.0 $\pm$ 0.4
Pituitary	3.1 $\pm$ 0.5	4.1 $\pm$ 0.6	2.6 $\pm$ 0.3	2.1 $\pm$ 0.4	2.9 $\pm$ 0.3	2.8 $\pm$ 0.5

Ca and Mg concentrations in the incubation medium were verified by automatic complexometric titration (Zinsser, Frankfurt).

## Results

Gyrus, internal capsule, basal ganglia, cerebellum, and pituitary which were incubated in culture medium with a low Ca concentration secreted PTH. In the 4th hour the secretion increased rapidly, and in the 6th hour it reached a maximum of approximately 200%–350% of the basal value in the 1st hour of 100%.

In the incubation medium with a high Ca concentration the PTH secretion decreased and in the medium with a normal Ca concentration the secretion remained unchanged (Table 1). After the addition of 1.25(OH)<sub>2</sub>D (100 ng/ml) to the medium the PTH secretion diminished from 100% to 70%. This effect could be reversed by adding 10 mM DB-cAMP which again stimulates the PTH secretion. The results are given in the Fig. 1.

## Discussion

Our results demonstrate a PTH secretion by gyrus, internal capsule, basal ganglia, cerebellum, and pituitary of sheep in vitro.

The effects of Ca, 1.25(OH)<sub>2</sub>D, and DB-cAMP on the secretion of PTH by the parathyroid glands were investigated. An inverse relationship between PTH secretion and Ca has been described by several authors [8, 9, 11]. Also the inhibitory effect of 1.25(OH)<sub>2</sub>D on the PTH release has been observed [2, 4, 7]. The presence of an adenylate cyclase system in the parathyroid glands cells and the regulation of the PTH secretion by cAMP have also been demonstrated [1, 13]. cAMP has been found in almost all types of animal and bacterial cells [10] while Sutherland et al. [12] showed its role as a second messenger (in the activation of different cell

types by specific first messenger), in particular peptide hormones. cAMP participates in cell activation including exocrine and endocrine secretion. Our results indicate that cAMP is also involved in the brain pituitary PTH secretion. In conclusion, the effects of Ca, 1.25(OH)<sub>2</sub>D, and DB-cAMP on the PTH release observed in this study indicate the existence of a classic calcium endocrine regulatory mechanism in CNS/pituitary. The secretion of PTH by CNS and pituitary suggest that this hormone may play a role as neurotransmitter or neuromodulator.

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Dr. S. Balabanova  
Universität Ulm  
Rechtsmedizin  
Oberer Eselsberg  
D-7900 Ulm (Donau)

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# **EXHIBIT 5**

# 1997 Program and Abstracts

Nineteenth Annual Meeting  
of the American Society for  
Bone and Mineral Research

Jointly Sponsored by  
The University of Texas Health Science Center  
at San Antonio Medical School

Cincinnati Convention Center  
Cincinnati, Ohio  
September 10-14, 1997

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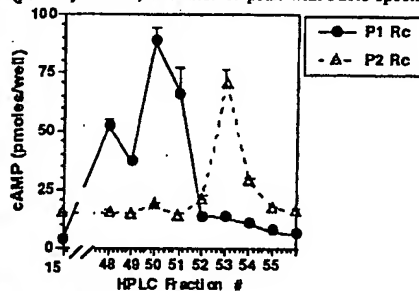
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## STUDIES ON A PUTATIVE PTH2 RECEPTOR-SELECTIVE LIGAND IN BOVINE HYPOTHALAMUS.

M. Mannstadt, H. T. Keutmann, S. Jusseume\*, H. Takasu\*, H. Jippner and T. J. Gardella. Endocrine Unit, Dept. of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.

The recently cloned PTH2 receptor, that recognizes PTH but not native PTHrP, might have a natural ligand that is different from PTH. Recently, evidence was provided for a PTH2 receptor-selective ligand in the brain (T. B. Usdin, *Endocrinology* 138: 831-834, 1997). To further characterize this putative new peptide ligand, we partially purified it from bovine hypothalamus following the procedure of T. B. Usdin with minor modifications. Following homogenization in acid-acetone and a C18 adsorption step, extracts were fractionated on a Sephadex G 50 column and then by reversed-phase HPLC. Screening for cAMP accumulation in COS-7 cells transiently expressing either the PTH/PTHrP receptor (P1Rc) or the PTH2 receptor (P2Rc) revealed two distinct peaks of activity, one peak with P1Rc-specific activity (possibly PTHrP) and another peak with P2Rc-specific activity (Fig.).



The purification and characterization of the latter peak of activity should lead to the identification of a new peptide ligand and its encoding gene.

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FUNCTIONAL PROPERTIES AND TISSUE DISTRIBUTION OF A PARATHYROID HORMONE TYPE 2 RECEPTOR (PTH2R) IN THE ZEBRAFISH (*Danio rerio*).

D. Rubin\*, J. Chen\*, L. Zou\*, M. Fishman\*, C. Bergwitz\*, and H. Jippner. Endocrine Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA, C.V.R.C., Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, and H.H.M.I. and Children's Hospital of Boston, Harvard Medical School, Boston, MA.

A zebrafish parathyroid hormone type 2 receptor (zebPTH2R) was cloned from a  $\lambda$ ZAP zebrafish kidney cDNA library. It showed significant overall amino acid homology to the human PTH2R (hPTH2R), 63% identity, but only 48-51% identity with the type 1 PTH/PTHrP-related protein receptor from several mammalian species and *Xenopus laevis*. Genomic Southern blot analysis using either the 5' untranslated or the tail exon of the zebPTH2R indicates that only a single gene is present. Wholemount *in situ* hybridization studies were employed to determine the tissue distribution of the zebPTH2R in the early zebrafish development. In two and three day old embryos, the zebPTH2R was abundantly expressed in brain, kidney, and vasculature.

Functional expression of this teleost zebPTH2R in mammalian COS-7 cells showed that cAMP accumulation was stimulated by analogs of human, rat, bovine, and chicken PTH, but not by PTHrP. This indicates that the zebPTH2R has a ligand specificity which is similar to that of the human PTH2R homolog. It is therefore likely that a PTH-like molecule exists in zebrafish, and possibly other teleosts which lack parathyroid glands. Further characterization of the zebPTH2R, and the identification of other zebrafish receptors for PTH and/or PTHrP, will enhance our understanding of the molecular evolution and physiology of these G protein-coupled receptors, and may lead to the isolation of teleost PTH-like ligands.

S370

## SPECIFIC IMMUNOLocalIZATION OF THE HUMAN PTH/PTHrP RECEPTOR IN CLASSICAL HUMAN TARGET TISSUES AND OVARY. M. Elizabeth Bruns, Mitch Scott\*, Jack Ladenson\*, Julia Iezzoni\*, Amy E. Adams, Larry J. Suva, James F. Ferguson II\*, Rogers M. Scanes\*, and David E. Bruns, University of Virginia, Charlottesville, VA, Washington University, St. Louis, MO, and Harvard Medical School, Boston, MA.

Parathyroid hormone (PTH) regulates calcium and phosphate metabolism via the activation of a specific G protein-coupled, membrane-bound receptor. This receptor also recognizes the related, yet evolutionarily distinct hormone PTHrP-related protein (PTHrP). In an effort to identify the sites of expression of the hPTH/PTHrP receptor protein, we prepared monoclonal antibodies (mAb) against portions of the receptor. mAb 3D1.1 was characterized in detail. This mAb recognizes protein(s) with apparent molecular weights of 80,000 and 160,000 daltons in Western blots of cell lysates of a human embryonic kidney cell line (HEK-293, clone C-21) stably expressing the human PTH/PTHrP receptor. The mAb does not recognize the recently identified hPTH2 receptor, which shares 51% amino acid sequence identity with the hPTH/PTHrP receptor, expressed in these cells. Binding of the mAb to the PTH/PTHrP receptor is inhibited by an excess of the PTH/PTHrP receptor immunogenic peptide, but not by the corresponding region of the hPTH2 receptor. Immunohistochemical analysis of a wide variety of fixed, paraffin-embedded tissues, demonstrated specific PTH/PTHrP receptor expression in kidney tubular and glomerular epithelium and in afferent and efferent renal arterioles, but not in glomerular endothelium. The most intense staining was in intervertebral disc chondrocytes. All vasculature studied was stained in both vascular smooth muscle and endothelium. Thus, mAb 3D1.1 positively stained classical PTH target tissues. Specific immunolocalization was also observed in human ovary with intense staining in the outer single cell layer of epithelium and the oocyte as well as in endocrine structures of both the preovulatory follicle (theca) and corpus luteum (theca and granulosa cells). These findings support the previous identification of hPTH/PTHrP receptor mRNA expression in the human ovary and identify the cell types expressing the receptor. The location of the hPTH/PTHrP receptor in human ovary strongly suggests a role for PTH and/or PTHrP in normal human gamete maturation and embryonic development.

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## PTH/PTHrP-RECEPTOR KNOCK-OUT MICE DEMONSTRATE ALTERED EPIPHYSEAL CARTILAGE AND ENDOCHONDRAL BONE FORMATION. W.J. Landis, D. Block\*, K.J. Hodgens\*, H. Kronenberg\*, and B. Lanske\*. Department of Orthopedic Surgery, Children's Hospital, Boston, MA, and \*Endocrine Unit, Massachusetts General Hospital, Boston, MA.

To examine possible structural-functional relationships in chondrogenesis for the parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) receptor, femoral and tibial epiphyseal growth plate cartilage and adjacent perichondrium from normal 18.5-day old fetal mice and their littermates ablated for the receptor gene were studied by microscopy. Whole legs were fixed in paraformaldehyde-glutaraldehyde, processed, and embedded in LR White resin. Some specimens were post-fixed in osmium tetroxide. Samples were sectioned at 1  $\mu$ m thickness and stained with toluidine blue for light microscopy. Tissue regions analyzed by electron microscopy were thin-sectioned (~80 nm) and stained with uranyl and lead salts. Growth plates of long bones from PTH/PTHrP-receptor knock-out animals, compared to wild-type, were considerably shorter; contained fewer proliferating and maturing chondrocytes and smaller extracellular volume; appeared with disordered transitions between proliferating, maturing and hypertrophic cells; and failed to form typical longitudinal cell columns and septae. Knock-out mice also were marked by previously unreported extensive glycogen deposits among proliferating, maturing, and hypertrophic cells; and by a pattern of calcification occurring at multiple sites in hypertrophic zones rather than normally along longitudinal septae. Perichondrium and its constituent cells appeared relatively indistinguishable in knock-out and control mice. These observations were similar to those found in separate studies of PTHrP knock-out mice and suggest that the effects of PTHrP on the structure of growth plate chondrocytes, their temporal and spatial developmental sequence, and their ability to support endochondral ossification are mediated by the PTH/PTHrP receptor.

# **EXHIBIT 6**

**Report on Board of Scientific Counselors' Review  
Dr. Ted Usdin  
March 5, 2001**

**Summary of Discussion and BSC Recommendations**

The panel congratulated Dr. Usdin on his achievements in successfully cloning TIP39, the ligand for the orphan PTH2 receptor protein. They thought he had made a good gamble in concentrating on this effort, and they described the result as phenomenal. It would be a significant loss to NIMH if he were to leave. They felt that Dr. Usdin had made good choices in deploying his limited resources and in taking calculated risks. The panel indicated that his proposal would probably not fair very well in a study section because there were few hypotheses and because he did not have a great deal of expertise in the neurobiological systems he was proposing to investigate; however, the panel expressed confidence that Dr. Usdin would nonetheless be successful in these projects because he is both thoughtful and a problem solver. For the same reason, the panel was reluctant to be prescriptive, preferring to let Dr. Usdin continue to exercise his good judgment in future experiments.

The panel rated the prior work as outstanding and the future work as excellent to high excellent. They enthusiastically recommended that Dr. Usdin receive tenure at NIH. In addition, they endorsed Dr. Usdin's interaction with the BMAP project, concurring that the effort will benefit both from Dr. Usdin's technical expertise and from his attention to detail. The panel supported an increase in Dr. Usdin's resources. They saw training with Dr. Usdin as a great opportunity for post-doctoral fellows, although they suggested that the fellows be encouraged to interact with other fellows in the Laboratory of Genetics.

***ad hoc* Reviewer Reports**

***Reviewer 1***

1. **Quality** – The quality of this research program is clearly very high. There is no question that Dr. Usdin's output would compare favorably with investigators at top research institutions especially when taken in light the relatively small number of personnel who have worked on his research program. Experimental designs and methods are clearly appropriate to provide unambiguous answers and indeed have done so in the prior research period. I would recommend support for the project be augmented in the view of past productivity and substantial amount of important new work that is proposed.
2. **Creativity** – The investigator has clearly demonstrated thoughtful risk taking to address the problem of identifying novel peptides and their receptors. It should be kept in mind that his attempt to find ligands for PTH2R could easily have failed. The proposals to study TIP39 and PTH2R in relation to nociception are ambitious. It will certainly take creativity and careful planning to plunge into the difficult field of peptides and pain research as he proposes.

# **EXHIBIT 7**



# Characterization of the human and mouse genes encoding the tuberoinfundibular peptide of 39 residues, a ligand of the parathyroid hormone receptor family

I A Hansen, O Jakob, S Wortmann, T Arzberger<sup>1</sup>, B Allolio and E Blind

Department of Medicine (Endocrinology), University of Würzburg, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany

<sup>1</sup>Department of Pathology, University of Würzburg, Würzburg, Germany

(Requests for offprints should be addressed to E Blind; Email: eberhard.blind@mail.uni-wuerzburg.de)

## Abstract

The polypeptide TIP39 (tuberoinfundibular peptide of 39 residues) is a potent activator of the parathyroid hormone (PTH)-2 receptor (P2R) and an antagonist of the PTH-1 receptor (P1R). To clarify its possible physiological function(s), we studied its interaction with the human P1R and P2R and examined the expression of TIP39 in man and mouse. To find out possible sites of this ligand interaction in the organism, we identified the genes encoding the TIP39 protein precursors of *Homo sapiens* and *Mus musculus* in the databases of the human and mouse genome projects respectively. We then obtained the full-length cDNAs of both species by RACE-PCR. The deduced TIP39 preprohormones consist of an N-terminal 30 amino acid (aa) signal peptide followed by a 29 aa TIP39 precursor-related peptide, an Arg-Arg processing site, and the actual 39 aa TIP39 sequence. The first 23 aa of the actual TIP39 sequence, thought to contain the P2R receptor activation site, are identical in man and mouse and thus phylogenetically conserved. By contrast, the 16 aa C-terminal portion showed a higher degree of diversity (75% aa identity). By using RT-PCR, TIP39 was found to be highly expressed in human central nervous system tissues, trachea, fetal liver, and, to a lesser degree, in human heart and kidney. Using *in situ* hybridization,

TIP39 mRNA expression was revealed in various areas of the mouse brain. In a homologous human cell model using human embryonic kidney 293 cells stably transfected with human P1R and P2R, human TIP39 did bind to P1R with moderate affinity ( $IC_{50} \sim 10^{-7}$ – $10^{-6}$  M), but showed higher affinity binding to P2R ( $IC_{50} \sim 10^{-8}$  M), comparable to the affinity of human N-terminal PTH (hPTH(1–34)) to this receptor. In P2R-transfected cells, the cAMP pathway was activated more efficiently ( $\sim 10$ -fold) by TIP39 as a ligand compared to hPTH(1–34). In P1R-transfected cells, only hPTH(1–34) but not TIP39 was able to elicit a cAMP response, but TIP39 was able to directly antagonize the cAMP-stimulating effect of hPTH(1–34) on this receptor. In conclusion, we could show a possible function of TIP39 for the human organism as a potent activator of P2R (e.g. in brain) as well as an antagonist of the action of PTH and/or PTH-related protein on P1R (e.g. in bone and kidney). The physiological role of TIP39 in calcium metabolism with regard to these actions remains to be determined. The tools developed in this work will allow us to investigate the possible role of TIP39 as a locally or systemically secreted ligand modulating the function of the PTH receptor family.

*Journal of Endocrinology* (2002) **174**, 95–102

## Introduction

The polypeptide TIP39 (tuberoinfundibular peptide of 39 residues) has been discovered recently in bovine hypothalamus (Usdin *et al.* 1999b) as a third member of the parathyroid hormone (PTH) ligand family, which now consists of PTH, PTH-related protein (PTHrP) and TIP39 (Usdin *et al.* 2000). Whereas PTH plays a major role in calcium metabolism and PTHrP regulates bone and cartilage development (among other functions), the biological function of TIP39 is largely unknown. It shows a limited homology with PTH, only 9 out of 39 residues are

identical in the bovine amino acid (aa) sequence (Usdin *et al.* 1999b, Piserchio *et al.* 2000). TIP39 is a potent activator of the PTH-2 receptor (P2R), the physiological function of which is also unknown at present (Usdin 2000). However, TIP39 also acts as an antagonist on the PTH-1 receptor (P1R), to an extent which varies in different species (Hoare *et al.* 2000). TIP39 has been isolated from bovine hypothalamus due to its capability of activating the P2R. Since the publication of this discovery in November 1999 (Usdin *et al.* 1999b), there are still only very few data about the possible function(s) of this peptide and where it might be expressed in the organism, besides

the hypothalamus. A possible function of TIP39 in this hypothalamic region has been suggested recently by experiments showing some influence on hypothalamo-pituitary axes (Ward *et al.* 2001).

To clarify its possible physiological function(s), we studied the interaction of human TIP39 in a homologous human model system with the human P1R and human P2R and examined the expression of TIP39 in man and mouse.

## Materials and Methods

### Cell lines, peptides and animals

The cDNA of P1R (kindly provided by Dr Harald Jüppner, Boston, MA, USA) and P2R (kindly provided by Dr T Usdin, Bethesda, MD, USA) were subcloned into the expression vector pCEP4 (Invitrogen, San Diego, CA, USA) and used to generate human embryonic kidney (HEK) 293 cells stably expressing the receptor proteins, as described (Blind *et al.* 1995). The cells were maintained in DMEM medium with Glutamax, containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin in a humidified atmosphere of 95% air/5% CO<sub>2</sub> at 37 °C. Human PTH(1–34) (hPTH(1–34)) was purchased from Bachem (Heidelberg, Germany). TIP39 was custom synthesized by Immundiagnostik (Bensheim, Germany) using the published aa sequence (Usdin *et al.* 1999b).

Adult male CD-1 mice were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). Animals were kept under standard laboratory conditions with a 12 h light:12 h darkness cycle. All efforts were made to minimize both the suffering and number of animals used and all procedures were performed according to the accepted standards of good animal care.

### Radioligand binding assays

Competitive binding studies were carried out in intact cell assays by displacement of the radioactive ligand [<sup>125</sup>I]-Nle<sup>8,21</sup>-Tyr<sup>34</sup>-rat PTH(1–34)-amide (NEN, Boston, MA, USA). Cells were grown for 3 days in 24-well plates to confluence, exposed to serum-free assay medium (DMEM containing 0.1% BSA and 1 mM Hepes) for 1 h and incubated in assay medium containing 50 000 c.p.m. radioactive ligand and various concentrations of unlabeled hPTH(1–34) or synthetic TIP39 at room temperature for 1 h. The cells were washed three times with ice-cold medium and then dissolved in 1 ml 1 M NaOH for measurement of cell-associated <sup>125</sup>I in a γ-counter.

### Measurement of total inositol phosphate (IP) turnover and of accumulated intracellular cAMP

The determination of accumulated total IPs was carried out in cells metabolically prelabeled with [<sup>3</sup>H]myoinositol

(Amersham, Freiburg, Germany) as described previously (John *et al.* 2001). To improve detectability of rather small degrees of activation of this pathway, the protein kinase inhibitors H-89 (30 µM) and GF 109203X (6 µM) (both from Bachem) were added, since we had shown previously that blocking protein kinases A and C with these substances resulted in an enhanced IP response (Blind *et al.* 1996).

To stimulate intracellular formation of cAMP, cells were incubated in 12-well plates with DMEM medium containing 1% BSA, 20 mM Hepes buffer and 1 mM 3-isobutyl-1-methylxanthine at 37 °C for 15 min together with the test substances. After removal of the supernatant, accumulated intracellular cAMP was extracted with 1 ml 95% ethanol, pH 3. After 2 h, the alcohol was removed by evaporation and cAMP measured by RIA (Beckmann Coulter, Unterschleissheim, Germany).

### Data analysis

Data for ligand-stimulated second messenger accumulation and inhibition of radioligand binding were analyzed using the software package Prism (GraphPad Software, Inc., San Diego, CA, USA), which was also used to calculate EC<sub>50</sub> and IC<sub>50</sub> values.

### Isolation of RNA from solid tissues and RACE

Total RNA was isolated by a commercially available modification (TRIzol; Invitrogen, Karlsruhe, Germany) of the one-step phenol/guanidinium thiocyanate method (Chomczynski & Sacchi 1987). Poly A<sup>+</sup> RNA was isolated using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany).

RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech, Alameda, CA, USA) following the manufacturer's instructions. One microgram of poly A<sup>+</sup> RNA was used as starting material. The following synthetic oligonucleotides were used as primers for RACE-PCRs: *Homo sapiens* 5'-RACE (5'-CTGCACGGTAGGGGACTGTGCGGGAAGCTGG-3'), *Homo sapiens* 3'-RACE (5'-ATGGAGACCCGCCAGGTGTCCAGGAGCCCT-3'); *Mus musculus* 5'-RACE (5'-AGGCGCAGTCGTCAGTTCGACAGGCTC-3'), *Mus musculus* 3'-RACE (5'-ATGGAGACCTGCCAGATGTCCAGGACGCCC-3'). RACE products were analyzed in 1.5% agarose gels. Isolated bands were cut out of the gel and agarose was removed using Ultrafree-DA spin columns (Millipore, Eschborn, Germany). The cDNAs were inserted into the PCRII-TOPO vector (Invitrogen) following the manufacturer's instructions. Sequencing was performed by Toplab (Martinsried, Germany).

### Labeling of RNA probes with digoxigenin-UTP (DIG-UTP) by *in vitro* transcription

*In vitro* transcribed RNA (cRNA) was synthesized from cDNA fragments cloned into the plasmid PCRII-TOPO

(Invitrogen) using T7 or SP6 polymerase respectively. Plasmids were linearized by digestion with an appropriate restriction enzyme. Reactions were performed according to protocols of the DIG RNA labeling kit (Roche, Basel, Switzerland).

#### *In situ hybridization on cryosections*

Total brains of adult mice were dissected, carefully frozen with dry ice powder and stored at  $-80^{\circ}\text{C}$ . Cryosections ( $10\text{ }\mu\text{m}$ ) were transferred to SuperFrostPlus microscope slides (Roth, Karlsruhe, Germany) and fixed for 15 min in 4% para-formaldehyde/PBS at  $4^{\circ}\text{C}$ . *In situ* hybridization was performed as described in the *Nonradioactive In Situ Hybridization Application Manual* (Roche Molecular Biochemicals, 2nd edition). The reactive structures were visualized by colorimetric reaction (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate). The specimens were mounted in Aquamount (Dako, Hamburg, Germany) and analyzed under the microscope.

#### *RT-PCR*

The reactions were carried out using total RNA ( $250\text{ }\mu\text{g}$ ) as template and the Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The PCR products were analyzed in 1.5% agarose gels stained with ethidium bromide. The following synthetic oligonucleotide pairs were used as primers for *Mus musculus*, 5'-CTGCACGGTAGGGGGTCCTGTAGGAGGCTGG-3' and 5'-AGGCGCAGTCGCAGTGGCAGAGGCTC-3', and for *Homo sapiens*, 5'-CTGCACGGTAGGGGACTGTGCGGGAAGCTGG-3' and 5'-CGCAGTCCGGAGCGCAGGGGCATGGTC-3'.

#### *Nucleotide and aa sequence analysis*

Genome searches were performed using TBLASTN at the NCBI databases (<http://www.ncbi.nlm.nih.gov/>); sequence similarities were studied using Clustalw at EMBL (<http://www2.ebi.ac.uk/clustalw/>).

## Results

#### *Characterization of the gene encoding TIP39*

A database search in the human genome using the aa sequence described in the literature revealed a TIP39 gene locus on the long arm of chromosome 19 at band 19q13.3. In the mouse genome a sequence derived from the NCBI mouse genome database was identified. Starting with templates of mouse and fetal human brain, we obtained human and murine cDNAs by RACE-PCR (submitted to GenBank and available under the GenBank accession numbers: AY037555 and AC073740). An alignment of

the nucleotide sequences of the human and murine cDNAs revealed 80% identity. The human cDNA consists of a 5'-untranslated sequence of 102 bp, an open reading frame of 300 bp, and 55 bp of 3'-untranslated sequence containing a polyadenylation signal (Fig. 1A and B). The genes of both man and mouse consist of two exons separated by an intron at corresponding sites. Exon 1 encodes 43 aa of the TIP39 precursor protein, exon 2 encodes the remaining 57 aa (Fig. 1C).

A computational analysis (Nielsen *et al.* 1997) of the 100 aa prepro sequence indicates that the first 30 aa most probably function as a signal peptide that directs the polypeptide chain to the endoplasmic reticulum. The TIP39 precursor also contains two possible cleavage sites (Arg-Arg motif with compatible adjacent residues). The first separates an intercalated peptide from the secreted peptide (Fig. 1D), the second is found at position 22/23 within the actual TIP39 sequence, suggesting that they may play a role in the processing of TIP39.

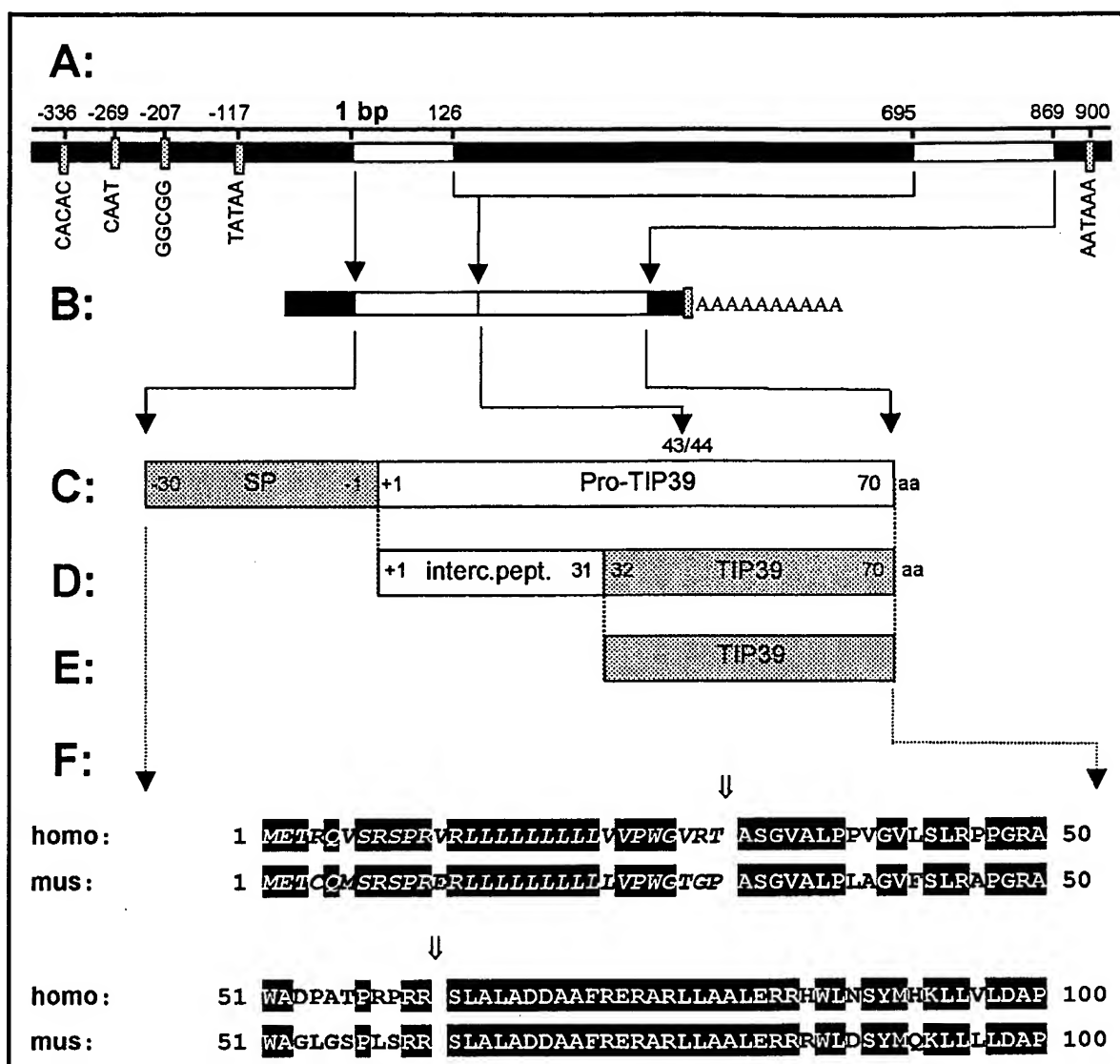
Comparison of the primary structure of the human and mouse preproTIP39 and the partially known bovine sequence using the Clustalw software shows a 100% identity between *Homo sapiens* and *Bos taurus* within the actual TIP39 sequence. Man and mouse share 79% overall identity and 89% identity within the secreted TIP39 peptide itself (Fig. 1F). The identities of the signal peptides comprise 77%, the intercalated peptides 68%. The actual TIP39 sequence shows the highest degree of identity in the N-terminal region.

#### *Distribution of TIP39 mRNA in man*

By RT-PCR we screened human tissues for TIP39 mRNA expression (Fig. 2). Using templates of fetal and adult tissues we obtained strong bands in fetal and adult brain, cerebellum and trachea. Furthermore, there was evidence for TIP39 mRNA synthesis in spinal cord, fetal liver, kidney and heart. No response was detected in adult liver, lung, placenta and adrenal gland.

#### *Expression of murine TIP39 mRNA in mouse brain*

To evaluate the expression pattern of TIP39 mRNA, a DIG-labeled RNA probe was constructed and the distribution examined by *in situ* hybridization on cryosections from mouse brain (Fig. 3). A wide range of hybridization intensities was observed, with many positive neurons throughout all regions of the nervous system. In the cerebral cortex and subcortical areas, e.g. septal nuclei and caudate-putamen, many neurons were densely labeled whereas the glia cell-rich corpus callosum remained unstained. In the cerebellum, hybridization signals were found in Purkinje cells, and in cells of the molecular layer (probably basket and stellate cells), but were nearly absent in the neuron-rich granular cell layer.

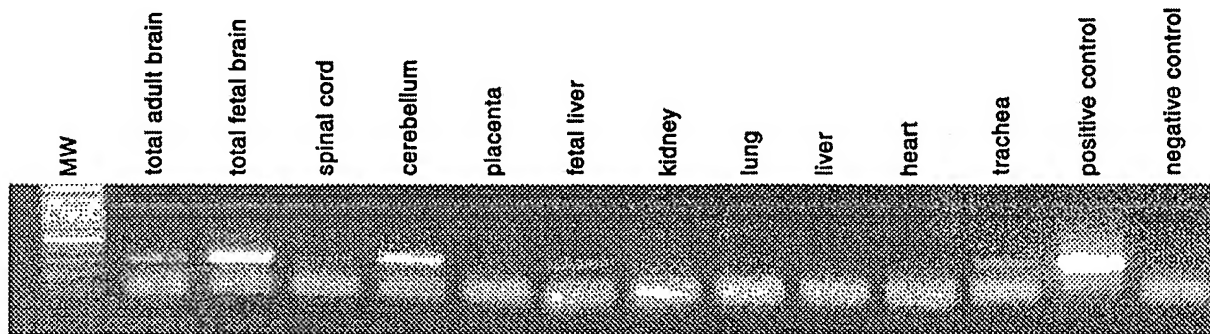


**Figure 1** Scheme of the human TIP39 gene transcription, translation and posttranslational modifications and comparison of the deduced TIP39 preprohormones of *Homo sapiens* and *Mus musculus*. (A) TIP39 gene; conserved sequences of the promoter region and the polyadenylation signal sequence are in gray boxes, exons are white boxed, untranslated regions and the intron are black boxed. (B) TIP39 mRNA; untranslated regions are black boxed. (C) TIP39 preprohormone; the hydrophobic signal peptide (SP) is gray boxed. (D) TIP39 prohormone; intercalated peptide (interc. pept.) is white boxed, TIP39 core region is gray boxed. (E) Finally processed TIP39 peptide. (F) Deduced TIP39 preprohormones of *Homo sapiens* and *Mus musculus*; identical aa are black boxed, the signal peptides are shown in italic letters, presumed cleavage sites are marked with an arrow.

#### Comparison of PTH and TIP39 actions on the human PTH receptors

We directly compared ligand binding of human TIP39 and hPTH(1–34) to the human P2R and P1R in an intact cell radioligand assay as described above. In both P1R-transfected and P2R-transfected cell lines, the peptides were able to displace the radioactive ligand (Fig. 4A and B). Non-specific binding was less than 20% of the total

amount of tracer bound. Untransfected HEK 293 cells showed no specific binding of the radioligand (data not shown). hPTH(1–34) did bind to both human PTH receptor-transfected cell lines with similar affinities (P1R:  $IC_{50}$  = 19 nM; P2R:  $IC_{50}$  = 16 nM). Binding affinity of TIP39 was moderate with P1R ( $IC_{50}$  = 333 nM). It showed higher binding affinity to P2R ( $IC_{50}$  = 11 nM), comparable to the affinity of hPTH(1–34) for both receptors (Fig. 4A and B).



**Figure 2** Specific distribution of TIP39 mRNA in various human tissues. The blot shows RT-PCR results obtained by amplification of a 300 bp cDNA fragment (upper band). The lower band represents excessive primer. A plasmid containing the full-length human TIP39 cDNA was used as a positive control. MW: molecular weight marker. The negative control reaction was performed by omitting template RNA.

The activation of the cAMP signaling pathway of P1R and P2R was measured by standard RIA after incubation with increasing concentrations of hPTH(1–34) and TIP39. The P1R could only be activated by hPTH(1–34) ( $EC_{50}=0.21$  nM), whereas TIP39 in concentrations of up to  $1\text{ }\mu\text{M}$  showed no detected increase in accumulated intracellular cAMP (Fig. 4C). In P2R-transfected cells, the cAMP dose–response curve was shifted about 10-fold to the left with TIP39 as a ligand, compared to hPTH(1–34) ( $EC_{50}=0.36$  nM and  $3.50$  nM respectively). The maximal increase in cAMP accumulation was similar with both peptides (Fig. 4D).

Additionally, the activation of the phospholipase C pathway was estimated by measuring ligand-induced IP hydrolysis. Incubation with  $3\text{ }\mu\text{M}$  hPTH(1–34) caused a strong increase in intracellular total IP (about 10-fold) in P1R-transfected cells and a small increase (about 2-fold) in P2R-transfected cells. TIP39, however, was not able to elicit a response of the IP signaling pathway in either cell line (data not shown).

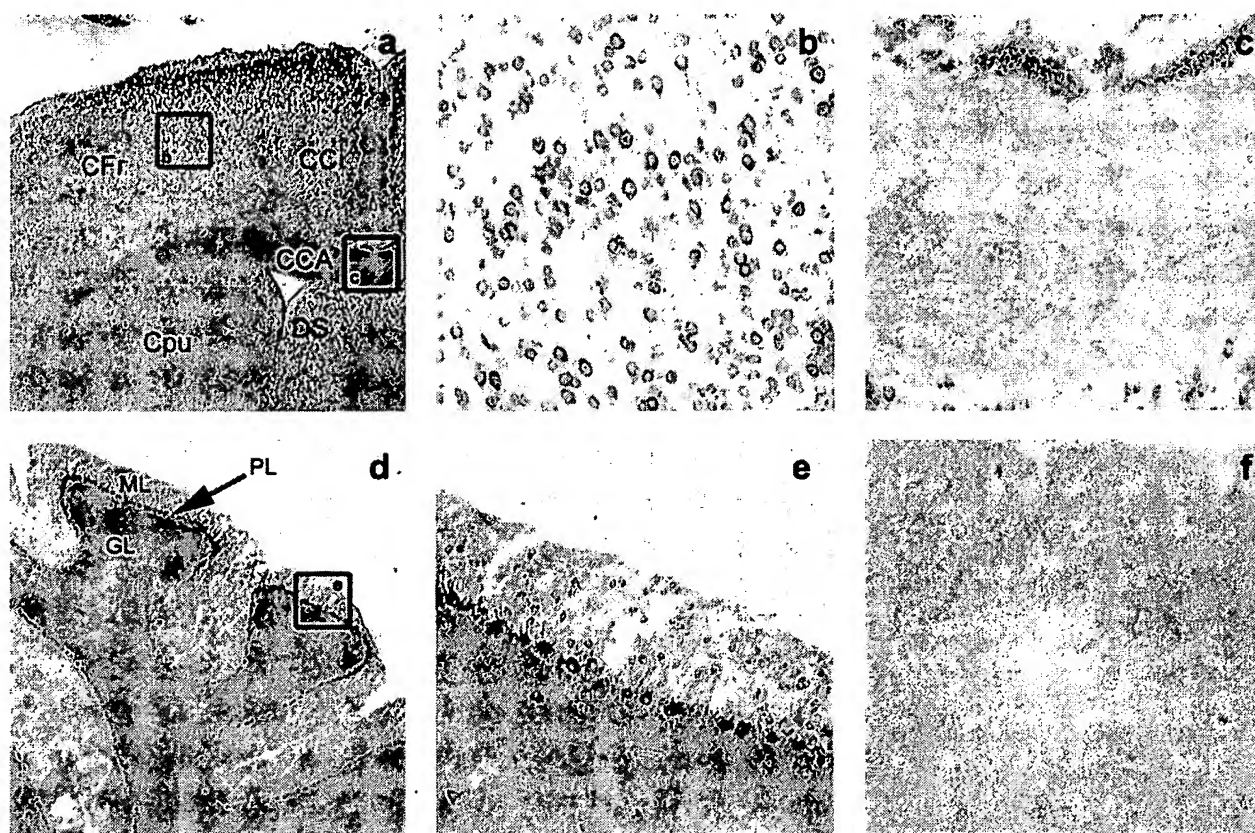
To test the antagonistic activity of TIP39 on P1R directly, we exposed P1R-transfected cells to increasing amounts of TIP39 up to  $10\text{ }\mu\text{M}$  in the presence of  $0.25$  nM hPTH(1–34). TIP39 almost completely inhibited agonist-induced cAMP accumulation with an  $IC_{50}$  value of half-maximal inhibition of approximately  $1\text{ }\mu\text{M}$  (Fig. 4E).

## Discussion

TIP39 may exert its biological function via two different mechanisms, either by inhibiting the action of PTH and/or PTHrP on P1R, or by acting independently on P2R. We have shown in our homologous human cell model system that human TIP39 acts as a competitive antagonist to the biologically active part of hPTH (hPTH(1–34)) on the human P1R. Additionally, we

showed for the first time that TIP39, unlike PTH on P1R, does not activate the IP signaling pathway in P1R or P2R. Our results are in accord with published results of similar model systems, often using heterologous systems with ligands, cells or receptors from other species, however (Hoare *et al.* 1999, 2000, Jonsson *et al.* 2001). It is thus conceivable that locally secreted TIP39 could inhibit the action of systemically circulating PTH, especially since the physiological levels of the bioactive part of this peptide are rather low in the circulation, in the range  $0.5\text{--}3$  pM (Gao *et al.* 2001). This would be especially relevant for the classic target organs of PTH in calcium homeostasis, bone and kidney. However, there are no published data on whether TIP39 is expressed in these organs. We were able to show that TIP39 mRNA is indeed present in the kidney, when looked at by RT-PCR. Whether TIP39 acts as a competitor of P1R or as an activator for P2R in these tissues is unclear at present. Some evidence comes from investigations with P2R, however. Although P2R mRNA was not detected by Northern blot in renal tissue (Usdin *et al.* 1995), Usdin *et al.* (2000) could show small numbers of cells near the vascular pole of kidney glomeruli to be P2R-positive by antibody staining and by *in situ* hybridization for P2R mRNA. These cells possibly belong to the juxtaglomerular apparatus, leading to speculations that their function might involve regulation of blood pressure as a target of a renin-releasing factor, which has been shown to be released from the hypothalamus and which is indistinguishable from TIP39 by size (Urban *et al.* 1992). We have no data yet on whether TIP39 is expressed in bone. It is also not clear at present whether P2R, as the other possible target besides the osteoblastic P1R, is present in bone, since no such study has been performed in this tissue so far. Attempts to detect P2R mRNA by RT-PCR in bone-derived cells have yielded equivocal results (Usdin *et al.* 2000).

Thyroid C-cells are another tissue linked to calcium metabolism where P2R was found to be expressed (Usdin



**Figure 3** Distribution of TIP39 mRNA in coronal sections of mouse brains visualized by *in situ* hybridization. A broad range of hybridization intensities was observed, with many positive neurons throughout all regions of the central nervous system. (a) Overview of a mouse brain cryosection ( $\times 12.5$ ). CCA, corpus callosum; CCI, cingulate cortex; Cpu, caudate-putamen; CFr, frontal cortex; DS, dorsal septal nucleus. (b) Inset of (a), showing cortical neurons in different layers stained with various intensity ( $\times 100$ ). (c) Inset of (a), showing absence of TIP39 mRNA in glia cells of the corpus callosum ( $\times 100$ ). (d) Overview of mouse cerebellum cryosection ( $\times 12.5$ ). GL, granular cell layer; PL, Purkinje cell layer; ML, molecular layer. (e) Inset of (d), showing distinct staining of Purkinje cells and of cells in the molecular layer, whereas staining was largely absent in the granular cell layer ( $\times 100$ ). (f) Cortical area corresponding to the area shown in (b) ( $\times 100$ ). *In situ* hybridization was performed using a DIG-labeled TIP39 sense RNA probe as a negative control ( $\times 12.5$ ).

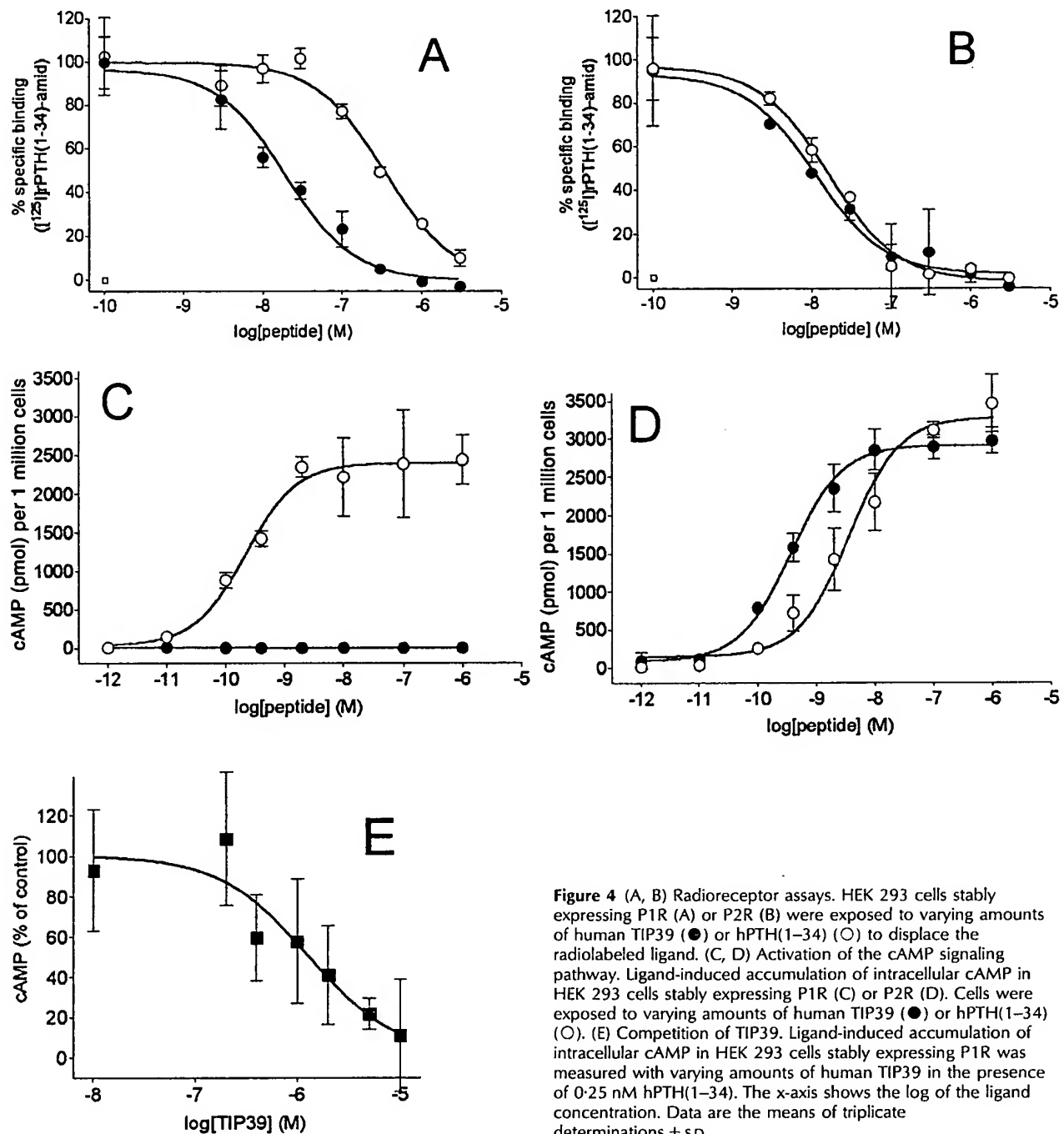
*et al.* 1999a). C-cells were specifically labeled by a P2R-specific antibody whereas the surrounding majority of follicular thyroid cells were negative. Since C-cells also express the calcium sensing receptor and respond with secretion of bioactive peptides upon an increase in serum calcium, there might be a link of P2R to the regulation of calcium metabolism. We have no data yet, however, on whether TIP39 is involved in this process.

As estimated by Northern blot, P2R is expressed at particularly high levels in brain. Unlike in peripheral tissue, TIP39 seems to be the single probable ligand of this receptor in brain, since investigators have failed to detect PTH mRNA in rat brain tissue (Usdin 1997), and since most regions of cerebral P2R expression would not be reached by PTH from the blood stream. The pattern of P2R distribution in the rat nervous system has been extensively studied recently (Wang *et al.* 2000). It shows a widespread expression, most often in discrete groups of

neurons, with an especially high level of expression in hypothalamic, limbic and sensory areas. The pattern of distribution of TIP39 expression found in our studies seemed to differ significantly, with TIP39 showing a distinct distribution pattern, which was, however, also widespread within various cerebral regions. A more detailed study is, therefore, needed to locate TIP39 expression in comparison to the P2R expression pattern.

We could detect TIP39 mRNA in human spinal cord. The rat P2R was shown to be expressed there, too, localized to the superficial dorsal horn, spinal trigeminal tract and nucleus. Because these areas receive their principal input from sensory neurons involved in pain perception (Usdin *et al.* 1999b), this colocalization might hint at a possible TIP39/P2R interaction modulating nociceptive function.

An analysis of the deduced aa sequence of the TIP39 preprohormones of man and mouse clearly shows the



**Figure 4** (A, B) Radioreceptor assays. HEK 293 cells stably expressing P1R (A) or P2R (B) were exposed to varying amounts of human TIP39 (●) or hPTH(1–34) (○) to displace the radiolabeled ligand. (C, D) Activation of the cAMP signaling pathway. Ligand-induced accumulation of intracellular cAMP in HEK 293 cells stably expressing P1R (C) or P2R (D). Cells were exposed to varying amounts of human TIP39 (●) or hPTH(1–34) (○). (E) Competition of TIP39. Ligand-induced accumulation of intracellular cAMP in HEK 293 cells stably expressing P1R was measured with varying amounts of human TIP39 in the presence of 0.25 nM hPTH(1–34). The x-axis shows the log of the ligand concentration. Data are the means of triplicate determinations  $\pm$  s.d.

features of secreted neuropeptides with a characteristic signal peptide for the secretory pathway and a dibasic cleavage site for subtilisin-like endoproteases to process the prohormone (Hosaka *et al.* 1991).

The overall structural features of the preprohormones of TIP39, PTH and PTHrP – signal peptide, intercalated

peptide and hormone – are identical. However, the intercalated peptide of TIP39 comprises 31 aa, and is much longer than the predicted intercalated peptides of PTH and PTHrP (8 aa). The overall aa sequence similarity between these three ligands is very low, whereas the tertiary structure of the portion interacting with P1R



seems similar (Piserchio *et al.* 2000). PTH, PTHrP and TIP39 thus seem to have only a distant phylogenetic relationship.

The N-terminal alpha helix of TIP39, which seems to be responsible for P2R activation, is identical in man and mouse, whereas the C-terminal alpha helix, which is important for receptor binding (Piserchio *et al.* 2000), differs in four aa positions, being apparently responsible for the different binding properties of murine and human TIP39 (Goold *et al.* 2001).

There is no experimental evidence yet that the predicted dibasic cleavage site found between the two alpha helices is functional. One might speculate that it is involved in the degradation and/or inactivation of TIP39, or that the possible cleavage products have bioactivity on their own. While it seems certain that TIP39 is in fact a secreted peptide, it is entirely unknown whether detectable amounts of TIP39 can be found in plasma and whether therefore this peptide could act systemically.

In conclusion, we could show that in the human organism TIP39 is expressed in various tissues and might function as a potent activator of P2R (e.g. in brain) as well as an antagonist of the action of PTH and/or PTHrP on P1R (e.g. in bone and kidney). The physiological role of TIP39 in calcium metabolism with regard to these actions, e.g. as a locally secreted ligand modulating the function of the PTH receptor family, remains to be determined, however. The tools developed in this work will allow further investigation of the function of this new ligand of the PTH receptor family.

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# **EXHIBIT 8**

# Identification and Characterization of the Zebrafish and Fugu Genes Encoding Tuberoinfundibular Peptide 39

MADHUSUDHAN R. PAPASANI, ROBERT C. GENSURE, YI-LIN YAN, YASEMIN GUNES, JOHN H. POSTLETHWAIT, BHASKAR PONUGOTI, MARKUS R. JOHN, HARALD JÜPPNER, AND DAVID A. RUBIN

Department of Biological Sciences (M.R.P., B.P., D.A.R.), Illinois State University, Normal, Illinois 61790; Endocrine Unit (R.C.G., Y.G., H.J.), Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114; Institute of Neuroscience (Y.-L.Y., J.H.P.), University of Oregon, Eugene, Oregon 97403; and Novartis Institutes for BioMedical Research (M.R.J.), Bone Metabolism Research, CH-4002 Basel, Switzerland

Although the PTH type 2 receptor (PTH2R) has been isolated from mammals and zebrafish, only its mammalian agonist, tuberoinfundibular peptide 39 (TIP39), has been characterized thus far. To determine whether zebrafish TIP39 (zTIP39) functions similarly with the zebrafish PTHR (zPTH2R) and human PTH2Rs and to determine its tissue-specific expression, fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*) genomic databases were screened with human TIP39 (hTIP39) sequences. A single TIP39 gene was identified for each fish species, which showed significant homology to mammalian TIP39. Using standard molecular techniques, we isolated cDNA sequences encoding zTIP39. The fugu TIP39 precursor was encoded by a gene comprising at least three exons. It contained a hydrophobic signal sequence and a predicted pro-sequence with a dibasic cleavage site, similar to that found in

mammalian TIP39 ligands. Phylogenetic analyses suggested that TIP39 forms the basal group from which PTH and PTHrP have been derived. Functionally, subtle differences in potency could be discerned between hTIP39 and zTIP39. The human PTH2R and zPTH2R were stimulated slightly better by both hTIP39 and zTIP39, whereas zTIP39 had a higher potency at a previously isolated zPTH2R splice variant. Whole-mount *in situ* hybridization of zebrafish revealed strong zTIP39 expression in the region of the hypothalamus and in the heart of 24- and 48-h-old embryos. Similarly, zPTH2R expression was highly expressed throughout the brain of 48- and 72-h-old embryos. Because the mammalian PTH2R was also most abundantly expressed in these tissues, the TIP39-PTH2R system may serve conserved physiological roles in mammals and fishes. (*Endocrinology* 145: 5294–5304, 2004)

IN MAMMALS, PTH is the major regulator of calcium-phosphate homeostasis (1), whereas PTHrP serves multiple roles, including a regulatory role in chondrocyte differentiation and proliferation, breast development, tooth eruption, and cardiac development (2, 3). Recently mammalian tuberoinfundibular peptide 39 (TIP39), a peptide distantly related to PTH and PTHrP, was purified from bovine brain (4). Subsequently cDNAs encoding mouse and human TIP39 were isolated from brain mRNAs (4–6). Although PTH has the capability of stimulating the human PTH type 2 receptor (PTH2R) expressed *in vitro* in HEK293 or COS-7 cells (7, 8), it has limited ability to stimulate cAMP accumulation in cells expressing PTH2Rs from other species, including zebrafish (9, 10). In contrast, all known PTH2Rs are activated with high efficiency and efficacy by human TIP39 (hTIP39), indicating that this peptide is the primary ligand for PTH2Rs, including the zebrafish PTH2R (zPTH2R) (11).

Studies of mammalian TIP39 indicate that the PTH2R-

TIP39 endocrine system is likely to have physiological roles that are distinct from those of PTH and PTHrP (12–14). Indeed, it has been hypothesized that the TIP39-PTH2R system may have physiological roles in the regulation of GH secretion (4, 15), pain perception (16), release of hypothalamic hormones (4, 12, 17), regulation of anxiety and depression (18), or cardiovascular and renal hemodynamics (13, 14, 19). Little is known about the biological function of TIP39 and its receptor in fishes. However, studies in nonmammalian vertebrates may help gain insights into its roles in mammals.

Human TIP39-PTH chimeras are potent antagonists at the human PTH1R (20), indicating that both peptides assume similar secondary structures. Furthermore, some human TIP39 analogs were shown to be potent antagonists at the human PTH1R (11). Study of nonmammalian TIP39 may further enhance development of potent agonists and antagonists at the PTH1R and PTH2R, and it is possible that such analogs may aid in improving treatments for diseases such as osteoporosis, hyperparathyroidism, and humoral hypercalcemia of malignancy.

Presently information regarding the potential role(s) of TIP39 and PTH2R during embryonic development are lacking, in part, due to difficulty of studying *in utero* development in mammals. In contrast, zebrafish transparency allows for monitoring of early development, thus allowing more readily than is possible in mammals studies to explore the biological roles of the TIP39-PTH2R system. Developmental studies in basal vertebrates, such as teleosts, may thus yield

Abbreviations: AUAP, Abridged universal amplification primer; BS/JK, bootstrap/jackknife; CNS, central nervous system; DIG, digoxigenin; fTIP39, fugu TIP39; GIP, gastrointestinal-inhibitory peptide; hpf, hours post fertilization; hPTH2R, human PTH2R; hTIP39, human TIP39; nPCR, nested PCR; PTH2R, PTH type 2 receptor; RACE, rapid amplification of cDNA ends; *shh*, sonic hedgehog; TIP39, tuberoinfundibular peptide 39; zPTH2R, zebrafish PTHR; zTIP39, zebrafish TIP39; zTIP39 SV, zTIP39 putative splice variant.

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insights that are not readily apparent in the more derived mammals. To facilitate such studies, we identified genomic DNA sequences encoding TIP39 from two phylogenetically distant teleost fishes, the zebrafish (*Danio rerio*) and the pufferfish (*Takifugu rubripes*) and then isolated a full-length cDNA and a presumed splice variant, which both encode zebrafish TIP39 (zTIP39). We furthermore defined the putative organization of the fugu TIP39 (fTIP39) gene; used synthetic zTIP39 to stimulate cAMP accumulation in COS-7 cells expressing the zPTH2R and its human homolog, hPTH2R; and determined the tissue-specific expression of both zTIP39 and zPTH2R by performing whole-mount *in situ* hybridization on zebrafish embryos. The use of two distantly related fishes not only allowed for a more robust statement on the conservation of TIP39 in fishes but also provided the necessary sequences with which to determine the phylogenetic relationship among the known members of the PTHrP-TIP39 family of ligands.

## Materials and Methods

### Identification of putative fTIP39 and zTIP39 transcripts

hTIP39 cDNA (5) was used as a probe to search the *T. rubripes* genome databases (<http://www.fugu.hgmp.mrc.ac.uk/blast/>, <http://134.174.23.160/compGenomics/>) for homologous sequences. A single genomic DNA sequence encoding fTIP39 (T004305 Scaffold\_4305) was identified and translated (<http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html>). The deduced amino acid sequence showed similarity to hTIP39. Subsequently this sequence was used as a probe to further screen the zebrafish (*D. rerio*) genomic databases ([http://www.sanger.ac.uk/Projects/d\\_rerio/](http://www.sanger.ac.uk/Projects/d_rerio/); <http://134.174.23.160/compGenomics/>). Two genomic sequences (zfshB-a2455h01.p1c, zfshI-a211c03.q1c) were identified, which showed significant sequence identity to the genomic DNA sequence encoded by fTIP39. Further searches were performed using the fugu Scaffold\_4305 of the Sanger zebrafish genome database ([http://pre.ensembl.org/Danio\\_rerio/](http://pre.ensembl.org/Danio_rerio/)) to identify sequences (ctg9592.2 and z06s017096) that contained a zTIP39-like gene. Gene-specific primers for zTIP39 were designed for RT-PCR and 5' and 3' rapid amplification of cDNA ends (RACE) reactions.

### RNA isolation, RT-PCR, RACE, and DNA sequencing

Total zebrafish RNA was obtained using the microRNA isolation kit (Promega, Madison, WI) as previously described (9, 21). To identify the 5' end of the cDNA encoding zTIP39, approximately 1  $\mu$ g of Dnase-treated total RNA from zebrafish was reverse transcribed using Omniscript II reverse transcriptase (Qiagen, Hilden, Germany) and a gene-specific reverse primer zTIP3ut#1 (5'-TTTTCCTATACCATCTTATA). One tenth of the RT-PCR product was used for an initial PCR consisting of reverse zTIP3ut#2 (5'-TTACAATTACTTGAATTAACACTAC), forward zTIP5ut#2 (5'-GAGTGTTAGAGAGAACTCTG), and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), with the following reaction profile: initial denaturation at 94 C for 3 min and 35 cycles with denaturation at 94 C for 1 min, annealing at 54 C for 1 min, polymerization at 72 C for 2 min, and final extension at 72 C for 10 min. A nested PCR (nPCR) using 2  $\mu$ l of the initial PCR product was performed using reverse zTIP3ut#2 (5'-TTACAATTACTTGAATTAACACTAC) and forward zTIP5ut#3 (5'-CATGGACGATTGCGAATTAG) following the same reaction profile. The 5' RACE amplicons were electrophoresed through a 2% agarose gel containing ethidium bromide, purified, ligated to pGEM-Teasy (Promega), and named zTIP39-5RACE/pGEMT (21) and used to transform *Escherichia coli* TOP10 cells (Invitrogen). Bacterial colonies were screened by PCR using gene-specific primers. Plasmids containing zTIP39 DNAs were purified using Concert miniprep (Life Technologies, Grand Island, NY) and sequenced according to the manufacturer's protocols (ABI, PerkinElmer Corp., Foster City, CA).

To identify the 3' end of the cDNA encoding zTIP39, total RNA was converted into cDNA using superscript II reverse transcriptase and an oligo-dTTP adapter-anchor primer (5'-GGCCACGCGTCGACTAG-

TACTTTTTTTTTTTTTTTT) containing unique restriction endonuclease sequences (Invitrogen). cDNA amplicons were amplified by PCR using forward zTIP5ut#2 and an adapter primer [abridged universal amplification primer (AUAP), 5'-GGCCACGCGTCGACTAGTAC] by following the same PCR profile described above except for an annealing temperature of 56 C for 1 min for 35 cycles. This reaction was followed by a nested amplification using forward zTIP5ut#3 and the AUAP adapter primer at an annealing temperature of 58 C for 40 cycles. Five microliters of the nPCR product was reamplified in a second nPCR using the above profile with the AUAP adapter primer and forward zTIPM (5'-CGTGATTGGAGCATTGAGATG). The 3'-RACE cDNAs for zTIP39 were isolated, subcloned into pGEM-Teasy and named zTIP39-3RACE/pGEMT, screened, and sequenced as described above. The translated zTIP39 cDNA sequence yielded an orthologous TIP39 peptide.

Kyte-Doolittle hydrophobicity plots for zTIP39 and fTIP39 amino acid sequences were generated ([http://bioinformatics.weizmann.ac.il/hydroph/cmp\\_hydrph.html](http://bioinformatics.weizmann.ac.il/hydroph/cmp_hydrph.html); <http://us.expasy.org/cgi-bin/protscale.pl>). Putative cleavage sites within the TIP39 precursors were predicted using SignalP V2.0b2 of the Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark (<http://www.cbs.dtu.dk/services/SignalP-2.0/>) (5). DNA sequence analyses and comparisons were performed using blast, translation, and alignment algorithms (<http://www.ncbi.nlm.nih.gov/BLAST/>, <http://www.searchlauncher.bcm.tmc.edu/seq-util.html>).

### Determination of putative intron/exon boundaries

The intron/exon structure of the fTIP39 gene was determined using Grail Experimental Gene Discovery Suite (Baylor College of Medicine Human Genome Sequencing Center, <http://www.searchlauncher.bcm.tmc.edu/seq-search/gene-search.html>) to search for putative introns, and the Splice Site Prediction by Neural Networks (Berkeley Drosophila Genome Project, [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)) to predict the locations of RNA splice sites.

### Peptide synthesis

The peptides, zTIP(1–39) and hTIP(1–39) (5), were synthesized at the Biopolymers Core Facility at Massachusetts General Hospital (Boston, MA) by a solid-phase method on a PerkinElmer model 430A and 431A synthesizer. All peptides were purified to homogeneity by reversed-phase chromatography, and amino acid sequences were confirmed by analysis of amino acid composition and amino acid sequence and mass spectroscopy.

### cAMP accumulation assays

COS-7 cells were transiently transfected with cDNAs encoding hPTH2R, zPTH2R, or zPTH2R #43–9 splice variant (zPTH2R SV) using Effectene reagent (Qiagen, Valencia, CA). After 72 h, cells were treated with 0–10<sup>−6</sup> M of either zTIP39 or hTIP39 in HEPES-buffered DMEM (pH 7.4) containing 2 mM isobutyl methylxanthine and 0.1% BSA for 60 min at room temperature. After rinsing, intracellular cAMP accumulation was measured by RIA as previously described (9, 22). The number of wells for each data point in each experiment was two (six total for all experiments). Thus, the data presented are the mean  $\pm$  SD of three combined experiments (as are the results shown in Table 2). The algorithm for curve fitting was a sigmoidal dose-response, and the analysis program used was GraphPad Prism (GraphPad, San Diego, CA).

### Zebrafish whole-mount *in situ* hybridization

TIP39 antisense RNA probe was produced by linearizing zTIP39-5'RACE/pGEMT with *MfeI* and then transcribing the cDNA using the digoxigenin (DIG) RNA labeling kit following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). The zTIP39 probe was used for whole-mount *in situ* hybridization on 24 and 48 h post fertilization (hpf) zebrafish embryos as described (23). A similar protocol was used to generate antisense zebrafish sonic hedgehog (*shh*) RNA for *in situ* hybridization on 48-hpf zebrafish embryos. After obtaining electronic images of the zTIP39 *in situ* hybridization whole-mount embryos, the embryos were embedded and sectioned at 10  $\mu$ m to determine the tissue-specific expression.

zPTH2R antisense RNA probe was produced by linearizing zPTH2R/pcrBlunt (#43–9) (9) with *Bam*HI and then transcribing the cDNA using the DIG RNA labeling kit as described above. The zPTH2R probe was used for whole-mount *in situ* hybridization on 48 and 72 hpf zebrafish embryos following the methods described by Jowett (24) and Nüsslein-Volhard and Dahm (25). The following reagents were used to reduce background: 150 mM malic acid in a 2% blocking reagent (Roche Applied Science) (24), 2 mM 1-phenyl-2-thiourea at 24-hpf (25, 26), and 1 mM levamisole added to the staining buffer (26). Following *in situ* hybridization, electronic images were taken on a Retiga (Burnaby, British Columbia, Canada) 1300 cooled CCD camera mounted on an SZX12 stereomicroscope (Olympus, Tokyo, Japan) using QCapture software on a G4 Power Mac (<http://www.Qimaging.com>).

### Sequence alignment and phylogenetic analyses

To examine the relationships between TIP39 and the family of PTH and PTHrP ligands, phylogenetic analyses were performed using all currently available species of these three peptides as previously described (5, 27). With the exception of equine PTH and bovine TIP39 for which precursor sequences were not available, complete amino acid sequences, which included the full-length prepro peptides, were used for alignment by T-Coffee and Dialign algorithms (28–30). T-Coffee and Dialign algorithms were used because they allow for a more accurate alignment, compared with ClustalW for sequences with less than 30% identity (28–30). The aligned amino acid sequences were subsequently entered into MacClade 4.0 (31) with manual adjustments as described (32) and analyzed using distance as the criteria by Neighbor-Joining and heuristic algorithms with PAUP version 4.0b10 (33). For each analysis, 10,000 bootstrap and jackknife replicates were performed in which the human gastrointestinal-inhibitory peptide (GIP) was used as the out-group, whereas secretin (human, pig, and mouse) and all known homologs of PTH, PTHrP, and TIP39 formed the ingroups.

## Results

### Identification of clones encoding zTIP39 and fTIP39

The cDNA sequence encoding hTIP39 was used as a probe to search fugu genome databases for similar sequences. A

single gDNA sequence (T004305 Scaffold\_4305) was identified which showed significant homology to exon 2 of the mammalian TIP39 gene and was therefore considered to represent a putative fTIP39 exon 2 (5, 6, 16). The fTIP39 exon 2 sequence was predicted to encode the prosequence and the mature secreted fTIP(1–39) (Figs. 1 and 2). Although a comparison between the teleost and mammalian signal peptide and leader sequences could not be performed due to a lack of sequence conservation, sequences beginning at the conserved Trp<sup>-11</sup> through the Ser<sup>+38</sup> or Ala<sup>+38</sup> residues showed, in comparison with hTIP39, an amino acid identity of 48% (24/49) and a similarity of 78% (39/49) (Figs. 1 and 2, and Table 1).

The fTIP39 gene structure was initially determined using Grail Experimental Gene Discovery Suite to search for putative introns, and the Splice Site Prediction program (Neural Networks) was used to predict the locations of RNA splice sites. Based on these analyses, the fTIP39 gene was predicted to have three exons (a presumed 5'-untranslated exon, and the coding exons 1 and 2), which would be similar to the organization of the murine and the human TIP39 genes (5, 6, 16) (Figs. 1 and 2). Differences between fTIP39 and mammalian TIP39 genes were restricted to the size of the introns and the length of the presumed fTIP39 exon 1 (Fig. 2). Once it was determined that fTIP39 and hTIP39 genes were homologous, genomic DNA encoding fTIP39 was used as a probe to tblastn search zebrafish genome databases for homologous sequences.

Two zebrafish genomic DNA sequences (zfishB-a2455h01.p1c, zfishI-a211c03.q1c) were identified, which showed 81% nucleotide sequence identity (100 of 122 nucleotides were identical despite two gaps encoding the peptide

FIG. 1. Nucleotide sequence of the fTIP39 gene. The putative mature mRNA encoding fTIP39 was deduced from zTIP39 cDNA sequences. Exonic regions are *capitalized*, nucleotides in flanking intervening DNA sequences are *lowercased*. The initiator ATG, with an upstream in-frame stop codon (tag), is shown in *bold* and represents the putative initial methionine and thus the start of the signal peptide. The splice donor and acceptor sites, with an intervening vertical bar (|), are *bold*. The stop codon has an \* below its corresponding nucleotide sequence, and the polyadenylation sequence is shown in *underlined lowercase letters*. Residues found in the putative preproprotein of fTIP39 are *capitalized* and indicated below their corresponding nucleotide sequence. Based on the cDNA sequence encoding zTIP39, the first residue of the translated mature TIP39 sequence is designated as +1 (N).

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tgcagattagaagaatacaagttcctcaaaacactccagtcaggctatatatattatgtgagctagaaa
aaaaatgctacacatagcagtggaatgcaaacgagcaggagcagaaaggcttccactgttttgactag
actggagaggcaccagagcaactggccgtacaacatcgtgtgggggttatactggagcattgctgctgct
presumed exon 5'UTR|intron
gctggaggacctcacctttaacctctctactacgatttacgactcaaatgaaacacag|gtgagaatcca
caaacggttttctaaatgaatccaagaacaacatgcaattagttttataaaagactttaaaaaaacttc
tcaatctggcaataaaaaatgggttcacatatattcttgtaattattctatgtcaacatacatatgcagc
atttaaatgttggtcctatcaaatgaaagttaattggataaactactgggtggcgtctcgaaactgggacttt
acatagttttcattgtaaaacacagaggcttttagtcatgttaagatgaactgttctcaagaagcaactt
ttccattttatcagaatgtgcaaacactgactgctttccatcaagtctcactcctgttactttgcatcc
intron|exon 1
tggatatgacttttgatgacatctgggtctccctcgactgtctcacag|gtgctcactcactcatttaacct
tcatttctatggcactttgaaaatgagcatataaaaggatttaaatatgcaactctgagatttgagctgtg
ttttctgctaaatgcttgcatgatttatctagaagtttagtttATGTCCTTCTCCAAAAGTTCTGACGATG
M S F S K S S D D
CCACAGCTGCCAAACAAGACAACCTGGGATGTGTTTTTCCCATCGCTCTTTTTCACAAATTGGAAAATTCAA
A T A A K Q D N W D V F F P S L F L H N W K I Q
ACAAATGTCAGCGCCCACTTGAAGCAGCGCGAGTAACAAGAGAGGTCTCGTGCAACAAGGGTGGCTTTT
T M S A P T L E A A A S N K R G L V Q Q G W L F
exon 1|intron
TGGACCCCAAGGATGGAGACAAG|gtaaccccatattagtgatgtttttgtttttctatgtgcaatattta
G P Q R M E T
caattttttctcatgtcaaatgataaaaaaagtgtcaacgcagtgctctggtgtgtagaatcttacttat
intron|exon 2
ttgttttttcttag|CTTGACGGAGTGTTCCTCAGGAATGGGCATCTCAGAGTGGTGGTATGGTGAAGA
S L D G V L P Q E W A S Q S G G M V K
+1
GGAACATGGTAATGGCTGACGATGCTGCCTTCAGAGAGAAGAGTAAGATGCTCACATCCATGGAGAGACAG
R N M V M A D D A A F R E K S K M L T S M E R Q
AAATGGCTGAACCTACATGCAGAAACTCTGGTGGTTAATTCAGCTtaggtaggcaaaaacagtagc
K W L N S Y M Q K L L V V N S A *
cagagagaagaatatatacatatgtaaaattccttaattgtacaaaaacatcatctcaaatgtttaa
ttgtgatttaactgtgtgatttaaaataaaa

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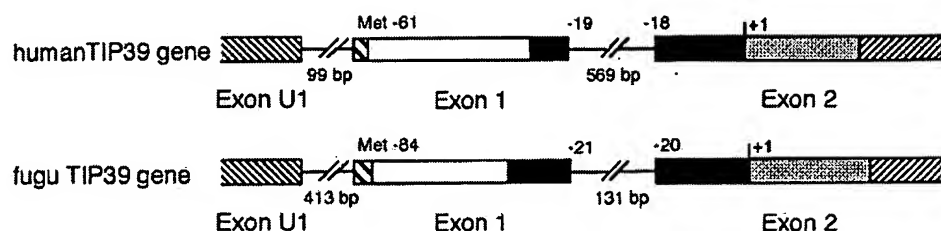


FIG. 2. Comparison of the structures for the human and fTIP39 genes. *Boxed areas* represent exons, and their names are shown underneath. The box representing exon U1 is open on the left side because the 5' end of this exon is currently unknown. *White boxes* denote presequences, *black boxes* denote the putative prosequences, *stippled boxes* denote the mature sequences, and noncoding regions are shown as *striped boxes*. The *small striped box* preceding the *white boxes* denote presumed untranslated exonic sequences. The positions of the initiator methionine based on the secreted peptide and the positions at which prosequences are interrupted by an intron are noted above the graphs. Intron sizes are noted below the //; and +1 denotes the relative position of the beginning of the secreted peptide.

TABLE 1. TIP39 nucleotide and amino acid sequence comparisons

	Exon U1 <sup>a</sup>	Exon 1 <sup>a</sup>	Exon 2 <sup>a</sup>	Total cDNA <sup>a</sup>	PreproTIP39 <sup>b</sup>	TIP(1–39) <sup>c</sup>
hTIP39	67	69	63	63	79/84	89/94
fTIP39	NSS <sup>d</sup>	NSS <sup>d</sup>	36	37	NSS <sup>d</sup>	51/92
zTIP39	NSS <sup>d</sup>	NSS <sup>d,e</sup>	32	33	NSS <sup>d</sup>	57/93

Comparison of the three exons encoding murine TIP39 (AC073763) with the corresponding nucleotide and amino acid sequences encoding human (h, AC068670), fugu (f, T004305 Scaffold\_4305), and zebrafish (z, contig ctg9592.2) TIP39. Protein comparisons were performed using the PAM250 algorithm with gap penalties existence = 13, and extension = 2; Nucleotide comparisons were performed using blastn matrix and gap penalties existence = 1, and extension = 0 (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>).

<sup>a</sup> Percent identity of nucleotides.

<sup>b</sup> Amino acid (AA) percent identity/percent similarity for the prepro sequence alone.

<sup>c</sup> AA percent identity/percent similarity for the secreted peptide.

<sup>d</sup> NSS, No significant similarity.

<sup>e</sup> Exon deduced by comparing fTIP39 genomic sequence to zTIP39 cDNA.

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catggacgatttgcgaattagcttggagtaaaacgtgagtgattggttttggagtcacacactggaaacaaag 75
agcttcacccacgcaacacacagctcaagaggaagaactactctgctaagATGGCTTTGTCCTTGCCCTCTCGTCC 150
                                     M A L S L P P R P
TGCCCTGTTGTTCTTAGTCTCATGAGTGTGACCTCATGSCATCGGCATTTCCTCCAGCCTCAGCTTCGACCTCT 225
A L L F L V L M S V T L M A S A F P Q P Q L R P L
GCAAAGTAACCTTGCCCTGCAATTGGTCAAGAAGACTCCAAAGGTGAGCAGTGGGAGGTGGTGTATCCATCCATCTC 300
Q S N L P A I G Q E D S K G E Q W E V V Y P S I S
GCTCCGTGATTGGAGCATTCAGATGCTGACCGCCCTCGATTGTTGGTGAGCTAAGACTGGGAGGAGCAGCTGGT 375
L R D W S I Q M L T A P D F G A A K T G R E Q L V
GGCAGATGATTGGCTCCCGCTCAGCCAATCACAGATGGAGGAGGAGCTGGTGAAGGGCTGGACGGGCGACTGGCC 450
A D D W L P L S Q S Q M E E E L V K G W T G D W P
TTCACGGGTGGGTCAACAGCAGAAAGAGAAATAGTGGTGGCAGATGACGCTGCGTTTAGAGAGAAGAGTAAGCT 525
S R V G H Q Q K R N I V V A D D A A F R E K S K L
GTTGACAGCAATGGAGAGACAAAATGGCTCAACTCTATATGAGAGCTCTTAGTAGTTAATTCAAAGtaatt 600
L T A M E R Q K W L N S Y M Q K L L V V N S K *
gtaattcttatgtataatgtataatataatagtgatagggaaaaatgcattttgtgtgtgggaaaaaaca 675
agaatttactcattacttcatgagtaaaacaattaaaggggtaggtcaactgagaattttaaattctgtcattgaat 750
tctaaaaaaaaaaaaaaaaaaaaa 773

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FIG. 3. Zebrafish cDNA and encoded TIP39 sequence. The amino acid sequence of the zTIP39 preprosequence is indicated below the corresponding nucleotide sequence. The putative secreted peptide is in **bold**. Nucleotides found in the putative preproTIP39 sequence are *capitalized*, nucleotides in the flanking 5' and 3' untranslated regions are *lowercased*. The ATG in **bold** represents the putative initial methionine and start of the signal peptide (accession no. AY306196), whereas the **boxed ATG** represents the putative initial methionine of a splice variant lacking nucleotides 125–187 (*underlined*, accession no. AY307076). The first residue of the translated mature zTIP(1–39) sequence tested in expression studies is **N**, the stop codon has an \* below its corresponding nucleotide sequence, and the putative signal for polyadenylation is *underlined*.

sequence Val<sup>-3</sup> through Ser<sup>+38</sup>) to the putative fTIP39 exon 2 sequence (Fig. 1). Further tblastn analyses were performed on the Sanger zebrafish genome database ([http://pre.ensembl.org/Danio\\_rerio/](http://pre.ensembl.org/Danio_rerio/)) identifying contig genomic DNA sequences (ctg9592.2 and z06s017096) containing a zTIP39-like gene with homology to fTIP39 and mammalian TIP39 (5, 6, 16). The presumed zTIP39 gene, although having a complete exon 2 and a 5' untranslated region sequence with limited homology to the 5' untranslated region of the fTIP39 gene (Figs. 1 and 2), did not appear to have an exon 1 sequence encoding a putative signal peptide. To determine whether

the TIP39-like zebrafish gene is expressed and to confirm the predicted intron-exon structure, zTIP39-specific primers for zTIP39 were designed for amplification by RT-PCR.

#### Teleost cDNAs encoding TIP39 and further definition of the gene structure

RT-PCR on total zebrafish RNA using zTIP39-specific primers amplified a 505-bp cDNA fragment, which corresponded to a 301-bp sequence of the presumptive fTIP39 exon 2 (T004305 Scaffold\_4305, Fig. 1). Subsequently, replicated and indepen-

mination codon for the fTIP39 sequence (Figs. 1 and 3). Several independent 5'-RACE nPCR using a 3'-reverse primer at the zTIP39 stop codon (nucleotide 580–604, Fig. 3) and a forward primer (nucleotides 1–21, Fig. 3) generated two PCR products, a 5'-RACE#14 (nucleotides 1–604) and the putative splice variant 5'-RACE#02 (zTIP39 SV) encoded by nucleotides (1–124) (188–604) (approximately 14% of all 5'-RACE products). Both cDNAs contained imperfect Kozak sequences (ACCAUGG)

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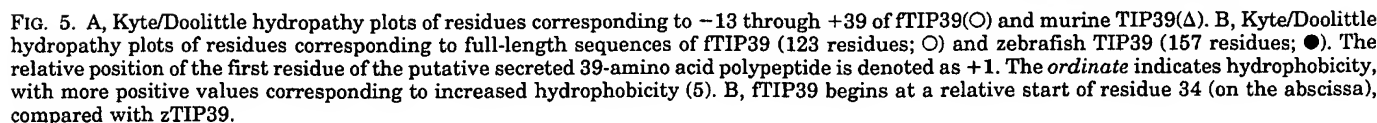
-91
  ↓
f TIP39 Mefskssddata aK
z TIP39 Ma LSLPPRPALLFLVLMSVTLMasafppqlrplqsnLPAIGqedsK
h TIP39 METRQVSRSPRVRLLLLLLLLLLVVPWGvrtASGVA LPPVGV
m TIP39 METCQMSRSPRERLLLLLLLLLVPGWgtgpASGVA LPLAGV
* * * * * : : * * *

f TIP39 qdnWDVFFPSLFLHNWKIQTMSAPTLEAAASNKRGLVQQGWL FGPORME
z TIP39 geqWEVVYPSISLRDWSIQMLTAPDFGAAKTGREQLVADDWLpLSQSQME
h TIP39 LSLR PPG
m TIP39 FSLR APG
* *

↓+1
f TIP39 TSLDGLVPQEWASQSGgmVKNRMVMADDAAFREKSKMLTSMERQKWLNSY
z TIP39 EELVGKGTGDWPSRVGHgqKRNI VVADDAAFREKSKLTTAMERQKWLNSY
h TIP39 RAWADPATPrpRRSLALADDAAFRERARLLAALERRHWLNSY
m TIP39 RAWAGLGSPlsRRSLALADDAAFRERARLLAALERRRWLDSY
* * * * * : : * * * : : * * *

f TIP39 MQKLLVNS 123
z TIP39 MQKLLVNSk 157
h TIP39 MHKLLVLDAP 100
m TIP39 MQKLLLLDAP 100
* * * * * : : * * *

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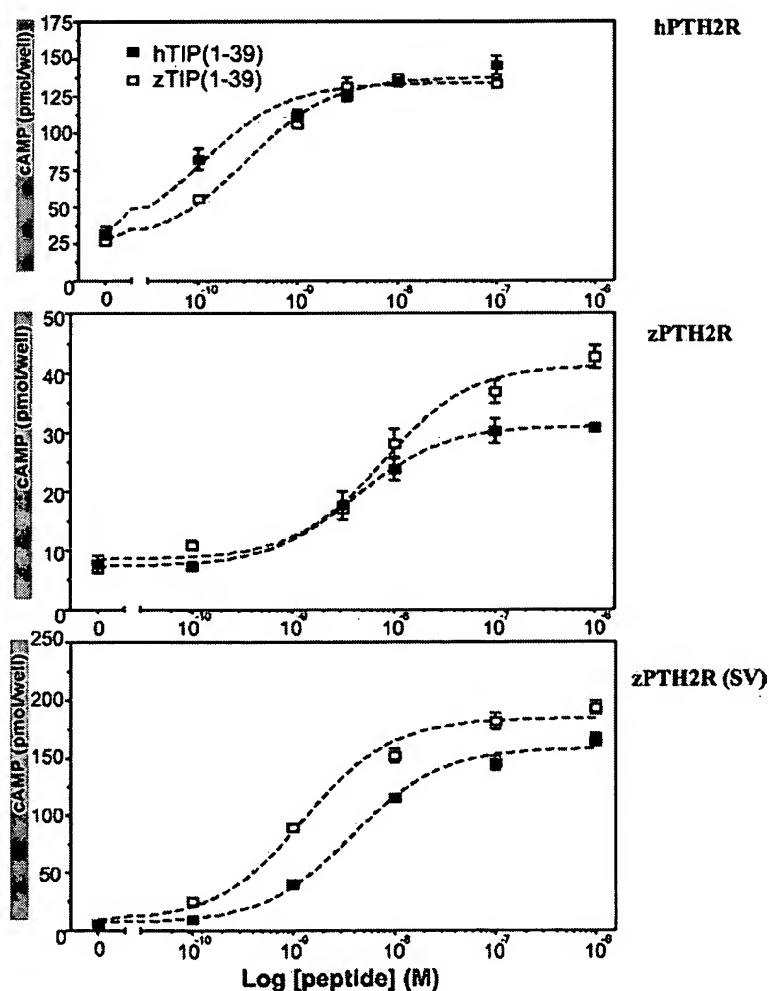
and differed in the lengths of their putative precursor sequences by 21 amino acid residues (nucleotides 125–187 encoding the signal peptide and mature peptide, Fig. 3) (6).

The results of these experiments suggested that there could be alternately expressed prepro-zTIP39 sequences, which are initiated at the third Met instead of the first Met and thus lack 63 nucleotides within the region encoding the poorly conserved signal peptide (Fig. 3). Although the putative splice variant is not missing any amino acids of the secreted peptide, we have not been able to identify typical splice donor and acceptor sites, which one would expect if this were a true splice variant, and it is thus conceivable that the putative splice variant is an artifact. The zTIP39 SV cDNA (accession no. AY307076) was 21 codons shorter than the full-length zTIP39 (accession no. AY306196), resulting in a putative precursor sequence of 97 amino acid residues instead of the 118 amino acid residues (Figs. 3 and 4). The putative secreted teleost TIP(1–39) peptides showed approximately 57% sequence identity with the mammalian TIP39 homologs, whereas the overall sequence similarity of the N-terminal precursor sequences showed little homology (Table 1). Although it appears that teleosts have maintained the inclusion of a mature peptide in the precursor sequence (amino acid residues –91 through –1, Fig. 4), the mature

peptide is poorly conserved, as is the signal peptide (amino acid residues –92 through –121). In addition, the mature peptide is much longer in teleosts (fTIP39: 72 amino acid residues; zTIP39: 91 amino acid residues) than the comparable sequence observed in mammals (human and murine TIP39: 31 amino acid residues) (6) (Table 1 and Fig. 4). Due to the lack of sequence conservation in the putative precursor (Fig. 4), compared with the presumed coding region of fTIP39 (123 amino acid residues) (Fig. 1), the zTIP39 SV of 136 amino acid residues showed a slightly higher nucleotide identity (49%), compared with the full-length zTIP39 of 157 amino acid residues (46%). In contrast, the zTIP(1–39) showed an 81% nucleotide sequence identity and 99% amino acid sequence similarity with the presumed fTIP(1–39) (Figs. 3 and 4, respectively).

The similarity among the coding regions for the mouse and fugu (Fig. 5A) and the two teleost (zebrafish and fugu) TIP39 sequences (Fig. 5B) is shown graphically in a hydrophobicity plot. Because of the size differences between the mouse and teleost TIP39 sequences, as well as a lack of conservation of the prepro sequence (Table 1), fTIP39 (amino acid residues –13 to +39) was aligned with amino acid residues –13 through +39 of mTIP39 (Fig. 5A), whereas the full length of fTIP39 (123 amino acid residues) was aligned with the full-

FIG. 6. cAMP accumulation induced by hTIP(1–39) and zTIP(1–39). COS-7 cells transiently expressing hPTH2R (A), zPTH2R (B), or zPTH2R SV (C) were evaluated for agonist-stimulated cAMP production (■, hTIP(1–39); □, zTIP(1–39)). Data are expressed as cAMP accumulation in picomoles/well and are shown as the mean  $\pm$  SD of three independent transfections;  $EC_{50}$ s were based on the data from three independent transfections.





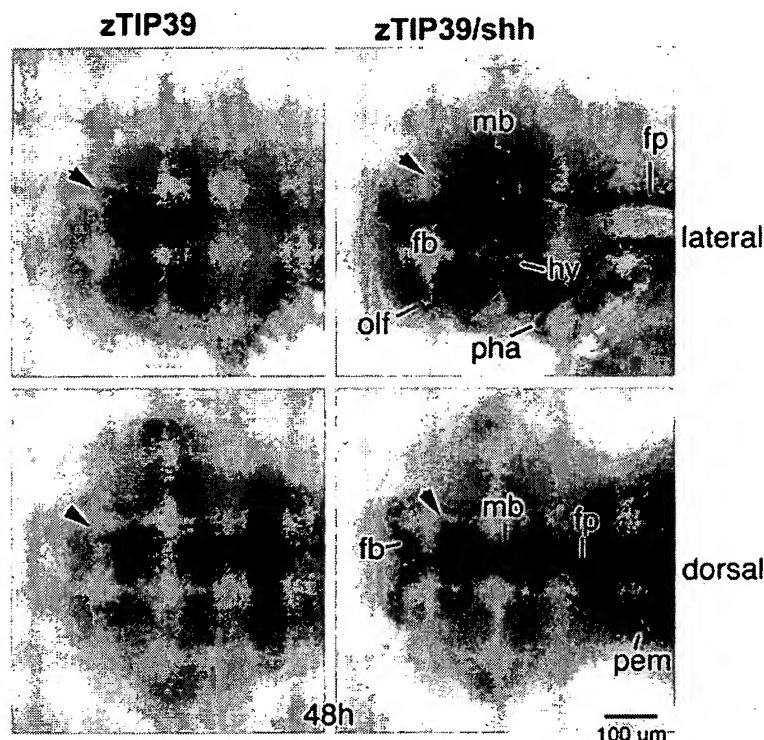
**TABLE 2.** Activation of COS-7 cells transiently expressing PTH2R by human and zebrafish TIP(1–39)

Ligand	hPTH2R		zPTH2R		zPTH2R (SV)	
	EC <sub>50</sub> (nM)	E <sub>max</sub> (pmol/well)	EC <sub>50</sub> (nM)	E <sub>max</sub> (pmol/well)	EC <sub>50</sub> (nM)	E <sub>max</sub> (pmol/well)
hTIP(1–39)	0.11 ± 0.04	134 ± 14	4.2 ± 0.3	31.1 ± 0.3	3.8 ± 0.7	159 ± 5
zTIP(1–39)	0.31 ± 0.07	138 ± 5	7.8 ± 2.0	41.4 ± 1.9	1.3 ± 0.3	184 ± 7
P	0.068	0.80	0.15	0.006 <sup>a</sup>	0.03 <sup>a</sup>	0.04 <sup>a</sup>

Accumulation of cAMP in response to human TIP(1–39) or zebrafish TIP(1–39) using COS-7 cells transiently expressing either hPTH2R, zPTH2R, or the zebrafish PTH2R splice variant 43–9 (zPTH2R SV). cAMP accumulation assays were performed as described in *Materials and Methods*. EC<sub>50</sub> and E<sub>max</sub> values represent the mean ± SD of at least three independent transfections. A pairwise Student's *t* test was performed between zTIP(1–39) and hTIP(1–39) at each receptor to determine the *P* values.

<sup>a</sup> Significant differences at *P* < 0.05 (60).

**FIG. 7.** Colocalization of zebrafish *shh* and zTIP39 assessed by whole-mount *in situ* hybridization double labeling. Whole-mount *in situ* hybridization of zebrafish embryos at 48 hpf using antisense cRNA encoding zTIP39 (left top and bottom, blunt arrow) and *zshh* (right top and bottom) with CNS locations indicated. The mRNA encoding zebrafish *shh* was expressed in the midbrain (mb), forebrain (fb), and hypothalamus midline, and the mRNA encoding zTIP39 in two lateral spots rostral and dorsal to the hypothalamus corresponding to a mb-fb border. Top two panels of lateral view, Rostral to the left, caudal to the right, dorsal above, and ventral below. Lower two panels of dorsal view, Rostral left and caudal right. fp, Floorplate; hy, hypothalamus; olf, olfactory pit; pem, posterior ectodermal membrane; pha, pharyngeal arch. All scale bars, 100 μm.



length zTIP39 (157 amino acid residues) (Fig. 5B). Thus, in Fig. 5B, fTIP39 is presented 34 amino acid residues to the right. The hydrophobicity plots for the available sequence indicated no hydrophobic leader sequence for fTIP39, which is comparable with that of mouse TIP(1–39) and zTIP39 SV. The remainder of the TIP(1–39) sequences showed nearly identical plots. In contrast to the other peptides, the full-length zTIP39 shows an initial hydrophobic leader sequence.

#### *In vitro* functional analysis of zTIP39 with the cognate zPTH2R

To compare the efficacy of zTIP39 with that of hTIP39, cAMP accumulation studies were performed on COS-7 cells transiently expressing the hPTH2R, zPTH2R, or zPTH2R SV #43–9 (Fig. 6, A–C, and Table 2). Synthetic hTIP(1–39) and zTIP(1–39) showed higher potencies with COS-7 cells transiently expressing the hPTH2R [EC<sub>50</sub>: 0.11 ± 0.04 nM hTIP(1–39) and 0.31 ± 0.07 nM zTIP(1–39)] than with cells expressing the zPTH2R [EC<sub>50</sub>: 4.2 ± 0.3 nM hTIP(1–39) and 7.8 ± 2.0 nM zTIP(1–39)]. However, cells expressing the zPTH2R SV were significantly better activated by zTIP(1–39) (EC<sub>50</sub>: 1.3 ± 0.3

nM) than by hTIP(1–39) (EC<sub>50</sub>: 3.8 ± 0.7 nM) (Fig. 6, A, B, and C, respectively, and Table 2). Although zTIP(1–39) and hTIP(1–39) showed similar efficacy in stimulating the hPTH2R (Fig. 6A and Table 2), zTIP(1–39) showed an approximate 3-fold enhanced potency in stimulating the zPTH2R SV, and a slightly enhanced efficacy (23 and 16%) in stimulating the zPTH2R and zPTH2R SV, respectively (Fig. 6, B and C and Table 2). The *in vitro* cAMP stimulation results thus suggested that, in addition to the significant structural conservation observed in the secreted TIP(1–39) (Fig. 3), there has been functional conservation of the ligand in vertebrates, at least when tested *in vitro* using a mammalian expression system.

#### *Expression analysis of zTIP39 and zPTH2R in zebrafish embryos*

Following established zebrafish protocols for whole-mount *in situ* hybridization using DIG-labeled RNA probes (23, 34), 24 and 48 hpf zebrafish embryos were used to assess TIP39 expression during development. At 24 hpf, zTIP39 was expressed at low levels generally in the central nervous sys-



tem (CNS), but by 48 hpf zTIP39 was expressed at high levels in tissues surrounding the hypothalamus (Fig. 7) as well as the developing heart (Fig. 8). To more specifically visualize structures in the central nervous system (Fig. 7), we performed double *in situ* hybridization experiments with the *shh* gene, which encodes a developmental signaling molecule and is expressed in the hypothalamus (35, 36). Whereas *shh* was expressed in the midline, which is consistent with its expression in the mammalian and fish hypothalamus, zTIP39 expression was detected in two lateral spots rostral and dorsal to the hypothalamus at a midbrain-forebrain border. Although our whole-mount *in situ* hybridization results indi-

cated that zebrafish express high levels of TIP39 mRNA during cardiac development (Fig. 8), previous studies indicated TIP39 to be expressed at low levels in the murine heart (5, 6).

The zPTH2R (#43–9) showed intense transcript expression by whole-mount *in situ* hybridization in the developing zebrafish CNS at 48 and 72 hpf (Fig. 9, A and B) as well as low level expression in the vascular tissue. These data are similar to those in mammals (13, 37, 38).

#### Phylogenetic relationships of teleost and tetrapod TIP39, PTH, and PTHrP

Full-length prepro amino acid sequences of PTH, PTHrP, and TIP39 were aligned and analyzed by distance methods (39) using human GIP as the outgroup as previously described (5). In addition, several secretin species were included in the analyses to determine and evaluate the relationships among TIP39, PTH, and PTHrP. Although the terminal branches showed minor variations (Fig. 10), depending on whether heuristic or Neighbor-Joining distance analyses were performed, all trees showed the same topology of groups, *i.e.* distinct clades for PTH, PTHrP, TIP39, and secretin. The current theory indicates that nodes showing bootstrap/jackknife (BS/JK) values above 95% are to be considered strongly supportive (40, 41). Thus, the BS/JK values supported the distinctiveness of a PTH-PTHrP clade (with PTH and PTHrP being sister groups), a TIP39 clade that is basal to the PTH-PTHrP clade, and a secretin clade.

#### Discussion

zTIP(1–39) and hTIP(1–39) are similarly efficacious and potent at the hPTH2R, zPTH2R, and zPTH2R SV (see Fig. 6). As such, a comparison of their amino acid sequences, as well as those of fugu and murine TIP39, may provide insights in identifying critical regions for receptor binding and activation. The TIP39 peptides are distantly related to PTH and PTHrP. Alignment of TIP39, with PTH and PTHrP, indicates the presence of two additional amino acids in the amino-terminal portion. Because PTH is a potent agonist, at least at the human PTH2R (42), it would have been surprising if these residues were critical for receptor activation. Whereas human and mouse TIP39 have identical residues at these first two positions, zebrafish and pufferfish have nonconservative substitutions.

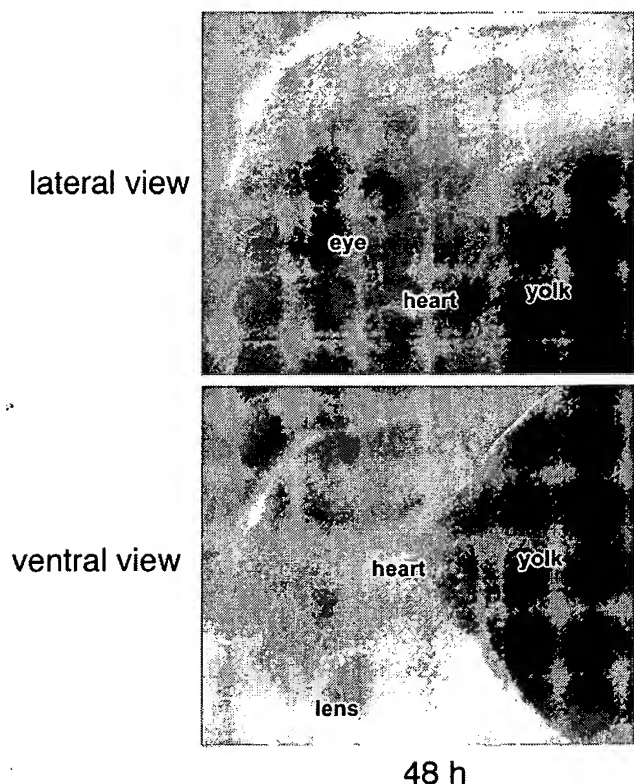
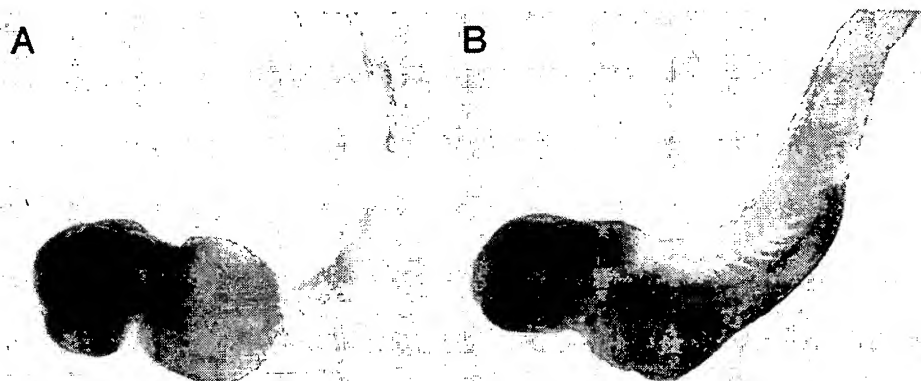


FIG. 8. Expression of mRNA encoding zTIP39 in developing cardiac tissues assessed by whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization of zebrafish embryos at 48 hpf using antisense cRNA encoding zTIP39 as a probe; TIP39 mRNA expression was observed throughout the developing cardiac tissue.

FIG. 9. Distribution of mRNA encoding zPTH2R in neuronal tissue assessed by whole-mount *in situ* hybridization. Whole-mount *in situ* hybridization of zebrafish embryos at 48 (A) and 72 hpf (B) using an antisense cRNA probe encoding zPTH2R (#43–9). PTH2R expression was observed throughout the developing zebrafish brain at 48 and 72 hpf.



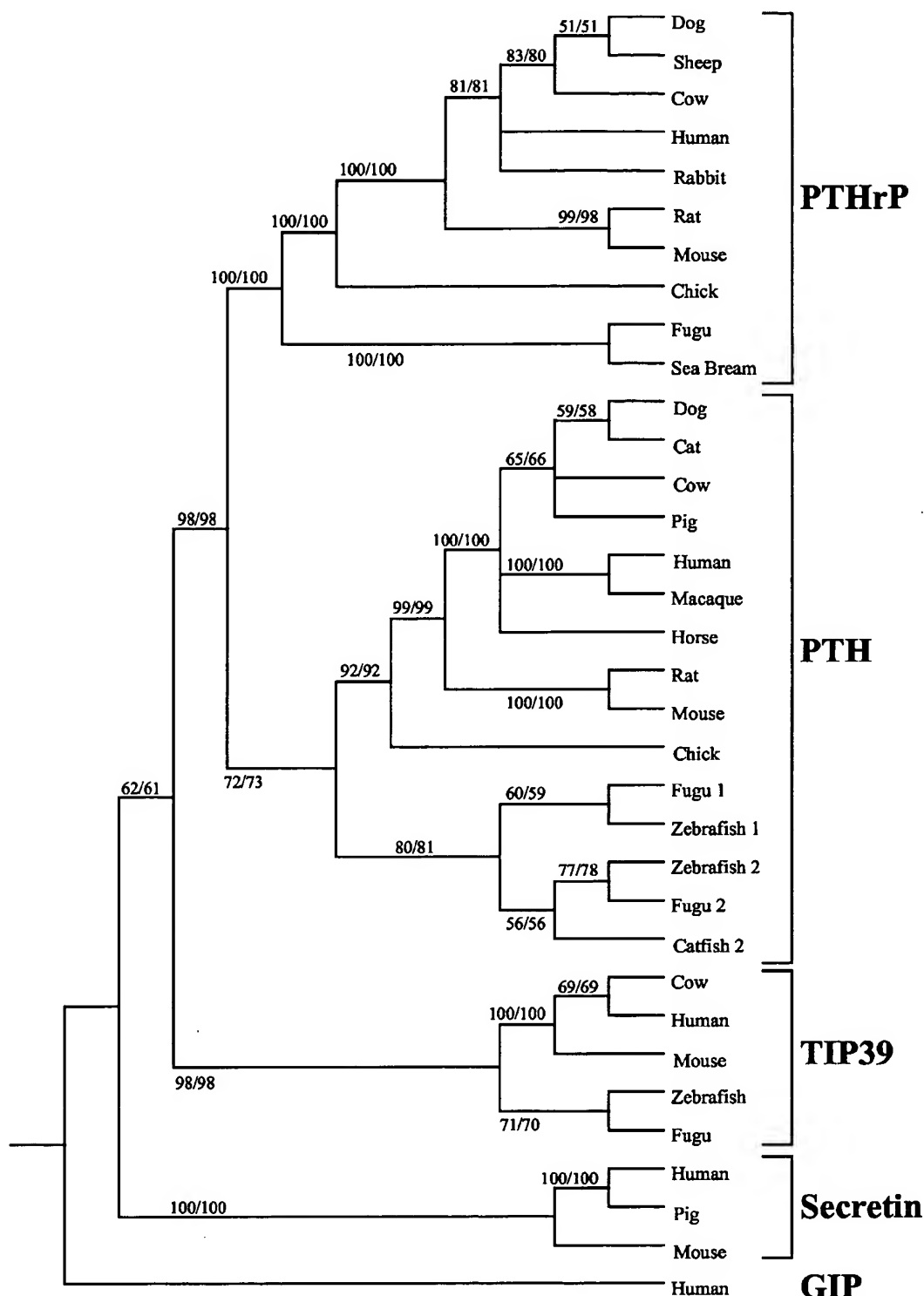


FIG. 10. Phylogenetic analysis indicating the evolutionary relationship among full-length prepro sequences of the TIP39, PTH, PTHrP, and secretin families of peptides. The phylogenetic tree is rooted with human GIP (5). The BS/JK values from 10,000 replicates indicate support of a given node in which 95% is considered to be significant (5, 40, 41). Using distance as the criteria, heuristic and Neighbor-joining phylogenetic analyses were performed. The overall topology of the trees were retained, although there were subtle variations in the terminal branches and minor variations in the tree statistics of the heuristic BS/JK phylogenetic analyses (tree length, 1099, consistency index, 0.850, and 166 parsimony-informative characters) and Neighbor-joining BS/JK phylogenetic analyses (tree length, 1101, consistency index, 0.848, and 166 parsimony-informative characters). All accession numbers used for the phylogenetic analysis have been previously listed (5), except for zTIP39 (AY306196), zTIP39 splice variant (AY307076), fTIP39, zPTH1 (AY275669), zPTH2 (AY275670), fPTH1 (59), fPTH2 (AY302221), and catfish PTH2 (BQ096842).

Structure-activity studies with PTH and PTHrP-based peptides have suggested that ligand residue 5 is critical for activation of the hPTH2R and that residue 23 is critical for binding to this receptor (8, 43). The TIP39 peptides from all four species do show conservation at these residues; however, as has been previously noted, the amino acid residue at position 7 in TIP39 (Asp) is different from that of PTH (Ile). Cross-linking studies with PTH-based peptides suggested that interactions in the activation domain occur at similar locations for hPTH1R and hPTH2R (44). Our own studies in the binding region of PTHrP suggest there are differences in the interaction site between PTHrP(1–36) analogs and the hPTH2R (45). The residues in the binding region of PTH and PTHrP, which were previously identified as intolerant to substitution (*i.e.* residues 23, 24, 28, and 31) (Ref. 46) are conserved between mammalian PTH and all of the currently isolated TIP39 ligands, suggesting that a similar mechanism of interaction occurs between these two peptides and the PTH1R. Indeed, chimeras between TIP39 and PTH indicate that hTIP39 binds to hPTH1R but does not activate this receptor, thus making it a potent antagonist (20). Furthermore, the arginine at residue 20, present in PTH and PTHrP in all known species, is also found in TIP39. Residues in positions 11 and 12, an important region for antagonist (47) and inverse agonist activity (48), are not conserved between TIP39 and PTH nor are they conserved in TIP39 across the species isolate thus far.

Overall, however, some critical regions for binding in PTH and PTHrP appear to be partially conserved in TIP39, whereas the activation domain showed less conservation between TIP39 and PTH. Furthermore, the results suggested that, even though the secreted forms of TIP39 appear to be highly conserved (Fig. 4), zebrafish PTH2Rs are capable of discriminating between teleost (zebrafish) and a tetrapod (human) TIP(1–39) ligands when tested in a mammalian COS-7 expression system. Because it is known that teleosts express two PTH-like peptides (27) and three receptors (9, 21), it appears that the teleost PTH- and TIP39-systems are more complex. This may be due to the evolutionary adaptability of teleosts in divergent environments when compared with mammals. Thus, to assess the physiological implications of TIP39 in teleosts and avoid potential confounding results, *in vivo* experiments may require species-specific (*i.e.* homologous) ligands.

Expression of zTIP39 mRNA was detected in a region that evolutionarily corresponds to the dorsal hypothalamic region (Fig. 7) (49, 50). Our whole-mount *in situ* hybridization results therefore suggested that teleost TIP39 may have similar roles as a neuropeptide as was postulated for mammals (5, 12, 17, 49, 51). The early expression of the zTIP39-PTH2R mRNA by whole-mount *in situ* hybridization suggested that the teleost system may have multiple roles in the developing brain (52, 53) and heart (54–56). Although it is unlikely that there is a direct developmental association between TIP39 and *shh*, by *shh* organizing the whole brain, of which TIP39 expressing cells are a part, it is conceivable that *shh* could influence where the TIP39 expressing cells will be localized (35, 49, 50, 57, 58).

The phylogenetic analysis indicated that TIP39 appears to be basal to PTH and PTHrP, thus indicating that TIP39 may be ancestral to PTH and PTHrP (Fig. 10). However, to confirm this statement, PTH-like sequences must be obtained from addi-

tional basal species. In addition, the PTHrP sequences showed a higher percentage of supportive BS/JK values when compared with PTH or TIP39 sequences. This would indicate that the PTHrP sequences retained many more invariant (*i.e.* conserved) residues that are necessary for function than PTH or TIP39, which showed much more sequence variation and thus the lower BS/JK values. This hypothesis is consistent with the recent finding of pairs of PTH expressed in zebrafish and fugu, which showed considerable divergence (27).

In summary, we have characterized the genes encoding zTIP39 and fTIP39 and isolated cDNAs comprising 157 and 136 amino acid residues encoding the full-length zTIP39 and a zTIP39 splice variant, respectively. Because the putative splice variant lacks a hydrophobic leader sequence, it is unlikely to be secreted. The zTIP(1–39) ligand activated the zPTH2R SV (#43–9) and zPTH2R expressed in COS-7 cells slightly better than the hPTH2R. However, it is presently unclear whether there is an associated *in vivo* conservation of physiological function(s). Phylogenetic analysis suggested that the TIP39 clade is basal to PTH and PTHrP, thus suggesting that TIP39 is ancestral to PTH and PTHrP. Finally, whole-mount *in situ* hybridization studies using probes encoding the zPTH2R and zTIP39 suggested that there are strong parallels in the neuroendocrine and cardiovascular expression of this system for mammals and fishes. Thus, zebrafish could make an excellent model to further investigate developmental roles of the TIP39-PTH2R system.

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Address all correspondence and requests for reprints to: David A. Rubin, Department of Biological Sciences, Illinois State University, Normal, Illinois 61790. E-mail: adavid@ilstu.edu.

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